REMARKS

Status of the Claims

Claims 26 and 156-162 are pending. Claims 1-25 and 27-155 were previously canceled without prejudice or disclaimer. Applicants reserve the right to pursue these claims in subsequent and pending applications. Claim 26 was withdrawn by the Examiner. Claims 156-162 are under consideration.

Amendments

The amendment to the specification was made to correct a typographical error. The amendment is supported by the text of the specification on page 20, lines 14-20, in conjunction with page 21, lines 11-13, of the as-filed specification. No new subject matter was introduced.

Inventorship

The Office stated that the instant application names joint inventors. Office Action, page 3. Applicant respectfully submits there is only one inventor, Donald Gullberg, of the instant application.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 161 and 162 were rejected under 35 U.S.C. § 112, first paragraph, as containing new matter. According to the Office, it is unclear where in the specification support can be found for the new limitations of claims 161 and 162 which were newly added in Applicant's amendment filed on September 7, 2006. Applicant respectfully traverses. The specification provides support for the newly added limitations in claims 161 and 162 throughout, including at page 1, lines 12-14; page 7, line 17, to page 8, line

19; page 20, line 14, to page 21, line 13; and in original claims 13-15, 18 and 19. Hence, claims 161 and 162 do not contain new matter.

Applicant therefore respectfully requests that the rejection of claims 161 and 162 under 35 U.S.C. § 112, first paragraph, be withdrawn.

Rejections under 35 U.S.C. § 103(a)

Claims 156-162 were rejected under 35 U.S.C. § 103(a) as being obvious over Gullberg et al. (Dev. Dyn. 204:57-65, 1995) (IDS Ref. No. C2), as evidenced by Velling et al. (IDS Ref. No. C5), in view of Alberts et al (1989) and US patent No. 6,046,316. Applicant respectfully traverses.

First, the premise underlying the Office's arguments is flawed. Contrary to the Office's assertion, Gullberg et al did not isolate integrin αmt or any other new integrin α subunit. Furthermore, Gullberg et al did not disclose a specific and identifiable new integrin α molecule, such as the integrin α11 of the instant invention. See Donald Gullberg's declaration, dated January 16, 2008 ("Gullberg declaration"), pages 2-3; and declaration by independent expert Staffan Johansson, dated January 16, 2008 ("Johansson declaration"), pages 3-4.

Second, the law provides that a conclusion of obviousness based on the combination of prior art reference teachings requires that a person of ordinary skill in the art would have had a reasonable expectation of success in arriving at the claimed invention. This is reflected in the recently published Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR International Co. v. Teleflex Inc.*:

> "The rationale to support a conclusion that the claim would have been obvious is that "a person of ordinary skill in the

> art would have been motivated to combine the prior art to achieve the claimed invention and that there would have been a reasonable expectation of success." If any of these findings cannot be made, then this rationale cannot be used to support a conclusion that the claim would have been obvious to one of ordinary skill in the art." Fed. Reg., Vol. 72, No.195, at 57534 (reference omitted; emphasis added).

Here, there was no reasonable expectation of success. In fact, the prior art not only did not provide any guidance regarding how to apply the teachings of Alberts et al to the isolation of integrin αmt or integrin α11, but actually taught away from such an approach based on technical problems. Furthermore, the expectation of success was strongly diminished by the recognition of the enormous complexity of the integrin family with respect to the large number of related genes and proteins as well as their complex expression patterns and regulation. See Gullberg declaration, pages 3-11; Johansson declaration, pages 2-6.

Gullberg et al did not *isolate* integrin αmt or any other new integrin

The Office contends that "Gullberg et al teach an isolated integrin subunit αmt obtained from G6 myoblasts and myotubes." Office Action, page 3 (emphasis added). Applicant respectfully disagrees. Gullberg et al did not isolate integrin αmt. See Gullberg declaration, page 2; Johansson declaration, pages 3-4. Gullberg et al merely identified a possibly new biochemical entity that interacts physically with integrin β1. Gullberg et al termed this entity integrin αmt based on certain biochemical properties that were reminiscent of known integrin α chains and the lack of immunological cross-reactivity with some of the known integrin α chains.

> "[A] protein migrating as 145 kD under non-reducing conditions was greatly induced in myotube cultures and co-precipitated with the β1 integrin chain (Fig.1). This induced protein was named integrin αmt (see Lack of Immunological

> Cross-Reactivity With Known Integrins)." Gullberg et al., page 58, left column , last paragraph.
>
> "Based on these data [of immunoprecipitations using antibodies to αv, α1, α2, α3, α4, α5, α6, and α9] we propose that the β1 integrin-associated protein induced in myotube cultures constitutes an integrin α-chain that we name αmt (mt from myotube)." Id. at page 59, first paragraph.

Hence, the integrin αmt of Gullberg et al is a biochemical entity that was characterized exclusively by immunological methods and that was not isolated or purified. Gullberg et al does not contain a single experiment designed to isolate or purify integrin αmt from cell extracts. This is important with respect to the Office's recitation of the teachings of Alberts et al (see below).

Gullberg et al did not disclose a *specific and identifiable* new integrin molecule, such as the integrin α11 of the instant invention

The Office further contends that the instant invention differs from the teachings of Gullberg et al. only by the recitation of defined integrin α11 domains, including the extracellular domain, the I-domain, the transmembrane domain, and the cytoplasmic domain, and of the heterodimer of claims 161 and 162. Office Action, page 3. Applicant respectfully disagrees.

The instant invention differs from Gullberg et al in many important aspects. One of the critical differences is that the instant invention discloses a novel integrin molecule, integrin α11, with a defined amino acid sequence and structure. Gullberg et al merely identified a biochemical entity whose sequence identity, structure, and relation to other molecules, including some of the integrin α chains, remained undefined. See Gullberg declaration, pages 2-3; Johansson declaration, pages 3-4 and 6. In this respect, Applicant notes that Gullberg et al did not investigate the relation between the

biochemical entity termed integrin αmt and integrins α8 and α10. Furthermore, it was well established at the time that integrins are a very complex family of proteins, in part due to alternative splicing. A case in point is integrin α7β1 which is developmentally regulated, at least in part, by alternative splicing.

> "Recent data show that α7β1 is subject to a developmentally regulated alternative splicing. In vitro analyses of α7β1 show a specific upregulation during differentiation of certain alternative splice variants." Gullberg et al., page 58, first paragraph (references omitted).

Hence, the biochemical entity termed integrin αmt by Gullberg et al may be identical, as defined by amino acid sequence, to integrins α8, α10, an alternatively spliced form of any integrin α chain, or an interaction partner of integrin β1 that is completely unrelated in sequence to integrins. In this respect it is important to note that integrin α10 has a very similar SDS-PAGE migration pattern and behavior under reducing conditions as compared to integrin αmt, and that integrin α10 also associates with the β1 integrin chain, as does integrin αmt. See Camper et al, J Biol Chem. 1998 Aug 7;273(32):20383-9, at abstract and page 20387. An integrin α chain may also adopt different conformations that can change its apparent molecular weight in SDS-PAGE. See Gullberg et al, page 61, right column, paragraph 2.

Because of the uncertainty as to the sequence identity of the biochemical entity termed integrin αmt, Gullberg et al could do no more than propose that integrin αmt was a new integrin α chain.

> "Based on these data we propose that the β1 integrin-associated protein induced in myotube cultures constitutes an integrin α-chain that we name αmt (mt from myotube)." Gullberg et al, page 59, first paragraph (emphasis added)

No definitive evidence was provided for this proposition. See Gullberg declaration, pages 2-3; Johansson declaration, pages 3-4 and 6.

Moreover, the biochemical entity termed integrin αmt was not the only potentially new β1 integrin-associated protein identified in the same set of experiments.

> "When immunodepletion was performed with antibodies to αv, α1, α3, α4, α5, and α6 integrin chains, the novel integrin-like protein was not co-precipitated with the integrin heterodimers formed by these α-chains (Fig.3). This excluded that the protein interacted with these different integrin heterodimers. When unbound proteins were precipitated with antibodies to β1 integrin, the novel protein was still precipitated. In addition, a band of lower molecular weight than the putative new α-chain was precipitated (see Discussion)." Gullberg et al., page 58, last paragraph (emphasis added).

> "The nature of the bands that were co-precipitated with αmt by anti-β1 integrin IgG from myotube cell extracts depleted with antibodies to α1, α3, α4, α5, α6, and αv antibodies is unclear, but might indicate the presence of other novel integrins on these cells, or might represent alternatively spliced variants of integrin α-chains not recognized by the depleting antibodies." Id. at page 60, last paragraph.

Hence, not only did Gullberg et al not disclose a specific and identifiable new integrin molecule with a defined sequence, as the instant invention does, but Gullberg et al also left open the relation between the biochemical entity termed integrin αmt, a similar entity with a slightly lower but similar molecular weight (see above), integrin α chains that were not included in the study, alternative splice variants of integrin α chains, and other, unrelated integrin β1 interaction partners. See Gullberg declaration, page 3; Johansson declaration, pages 3-4. In fact, the Applicant was never able to succeed in cloning an integrin α11 cDNA from a G6 cell cDNA library, arguing against a sequence identity between integrin αmt and integrin α11. See Gullberg declaration,

page 6; Johansson declaration, page 6; see also previously filed declaration by Donald Gullberg, dated November 10, 2005.

The new integrin of the instant invention, integrin α11, is deemed identical to integrin αmt only with respect to a very limited set of biochemical properties but not with respect to amino acid sequence or structure.

> "Based on similar SDS-PAGE migration patterns, similar behavior under reducing conditions, association with β1 integrin chain, and upregulation during in vitro differentiation of human fetal myoblasts, the present data show that α11 integrin is identical with αmt." Specification, page 25, lines 32-37.

In this respect, Applicant also refers to previously filed declarations by Donald Gullberg and by Teet Velling, dated November 10, 2005, and November 15, 2005, respectively. Applicant encloses copies of these declarations for the Examiner's convenience.

In sum, it is clear that Gullberg et al did not disclose a *specific and identifiable* new integrin molecule. Based on biochemical data alone, i.e. in the absence of any sequence information, a clear distinction of a "new" integrin molecule from other known integrin molecules was simply not possible. This notion was clearly expressed by an editor of a major peer reviewed journal in a rejection of a manuscript that had identified a novel integrin α chain by biochemical means only. See Johansson declaration, pages 4-5 and Appendix 3. As a result, one of ordinary skill in the art clearly would not have been motivated to attempt to determine the amino acid sequence of the integrin αmt taught by Gullberg et al based on this information alone.

One of ordinary skill in the art would have had no reasonable expectation of success

The Office further argued that based on the combined teachings of the references one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention, therefore rendering the invention obvious. Office Action, page 5. Applicant respectfully disagrees for several reasons.

1. Alberts et al did not provide an obvious path to the instant invention

The Office argued that it "would have been obvious to to one of ordinary skill in the art at the time of the invention was made to determine the amino acid sequence αmt subunit taught by Gullberg et al using the genetic engineering techniques as taught by Albert et al." Office Action, page 4. Applicant respectfully disagrees. See Gullberg declaration, pages 9-11.

The techniques referred to by the Office require that a protein is purified to homogeneity by an undefined series of chromatographic steps. See Alberts et al, page 174, paragraph 4. Only if a sufficient quantity of pure protein is obtained, the N-terminal amino acid sequence can be determined and a cDNA cloned. See Alberts et al, page 262, paragraph 4. As discussed above, Gullberg et al did not isolate or purify the biochemical entity termed integrin αmt. Gullberg et al does not contain a single piece of data that would allow an assessment of the purity or the abundance of integrin αmt. See Gullberg declaration, pages 9-10. Gel bands derived from metabolically labeled or surface labeled cells are not sufficient to make such as assessment. Furthermore, Gullberg et al do not disclose any biophysical properties of integrin αmt, except for its size and interaction with integrin β1, that would allow to design a purification strategy.

In addition, the detectability of integrin αmt varies greatly with the differentiation state of G6 cells. See Gullberg et al, page 58 and Figure 1.

In the absence of any knowledge of the structure of a protein of interest, its biophysical properties, and its abundance in the cell source of choice, it was anything but an obvious path to the cloning of its cDNA.

> "[B]ecause proteins vary in size, charge, and water solubility, no single method can be used to isolate all proteins. To isolate one particular protein from the estimated 10,000 different proteins in a cell is a daunting task that requires methods both for *separating* proteins and for *detecting* the presence of specific proteins." Lodish et al., Molecular Cell Biology, 3rd Edition, 1995, at page 88.

Gullberg et al did not provide any guidance on the methods for separating integrin αmt from the other estimated 9,999 proteins in the cell.

A suitable detection method must be highly specific, simple, fast, and sensitive enough that only a small proportion of the available material is consumed. See Lodish et al., Molecular Cell Biology, 3rd Edition, 1995, at page 96, paragraph 4. Neither did Gullberg et al provide any guidance as to a suitable method for detecting integrin αmt during a successful purification scheme.

Gullberg et al even indicated explicitely that a cloning strategy as described by Alberts et al would be problematic.

> "Further biochemical characterization of αmtβ1 presents some problems because of limited availability of human fetal week 10 muscle. Attempts to perform immunoaffinity purification on anti-β1 integrin columns from rat or mouse embryos will also present problem since αmtβ1 has similar molecular weight as α2β1 and α9β1." Gullberg et al, page 61, paragraph 2.
>
> "In a separate approach we are generating monoclonal antibodies to G6 cells. Initial attempts have generated

> muscle specific monoclonal antibodies but no antibodies reactive with αmt. In the absence of immunological tools we have no information about the distribution of αmtβ1 on non-muscle cells." Id.

Thus, Gullberg et al not only did not provide any guidance as to suitable methods for separating and detecting integrin αmt for purposes of purifying sufficient quantities of pure protein for partial sequence analysis, but actually taught away from such an approach. Therefore, it is clear that there would have been a very low expectation of success using this approach.

The potential difficulty and non-obvious nature of puryfing a sufficient quantity of a protein of interest in sufficient purity for successful partial sequence analysis was born out by the fact that the Applicant tried, but ultimately failed, to achieve such a purification of integrin αmt. See Gullberg declaration, pages 6 and 10.

2. The complexity of the integrin family strongly diminished the expectation of success

The enormous complexity of the integrin family, in particular with respect to its large number of related genes and proteins, its complex expression patterns, and its complex regulation, was widely recognized at the time of the invention. See Gullberg declaration, pages 3-4; Johansson declaration, page 3. Eleven (11) integrin α chains that associate with integrin β1 had been identified and characterized. Specification, page 1, lines 24-26. Furthermore, it was generally accepted that the identification of integrin proteins was complicated by the occurence of alternative splicing of integrin chains. This notion is expressed, for example, by Ziober et al:

> "Additional complexity of integrin regulation is exemplified by that fact that integrin subunits can be alternatively spliced. This alternative splicing has been shown to occur not only in

> a number of mammalian α and β subunits but also in Drosophila integrin receptors. Such splicing, which generally takes place in the cytoplasmic and the extracellular domains, can give rise to a number of subunit isoforms with diverse functional properties.... Investigating the function of this receptor [α7β1] has been complicated by the number of alternatively spliced isoforms of the α7 subunit. In the cytoplasmic domain, two isoforms, A and B, are expressed in a developmentally specific manner in skeletal muscle. Alternative splicing, in the extracellular domain of this subunit, also produces two muscle-specific developmentally regulated isoforms, designated XI and X2.... Furthermore, we show that activation of this isoform [α7-X1] is regulated in a cell-specific manner." Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34, at page 1724.

Moreover, it was well established that integrins are expressed in a cell type-dependent, differentiation state-dependent, and species-dependent fashion. See Gullberg declaration, pages 3-4; Johansson declaration, pages 5-6. This also applied to integrin αmt which was identified in human myogenic cells (G6 myotubes) but was undetectable in myogenic cells from rat or mouse. Gullberg et al, page 58, right column, first paragraph. Furthermore, integrin αmt protein levels were greatly dependent on the differentiation state of G6 cells. Gullberg et al, abstract, page 58, paragraph 3, and Figure 1.

Finally, the activation, posttranslational modification, and even sequence of various integrins was known to depend on cell type and differentiation state. See, for example, Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34.

> "The expression pattern of the laminin-binding α7β1 integrin is developmentally regulated in skeletal, cardiac, and smooth muscle." Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34, at Abstract.
>
> "[A]ctivation of certain integrins is dependent on cell-type specific regulation that may be due to posttranslational modifications, variations in the sequences of the α or β

> subunits, and/or the activation of different signal transduction molecules." Id. at 1723-4.

Three approaches were generally used in the art to clone the cDNAs encoding new integrin α chains. See Gullberg declaration, pages 4-6; Johansson declaration, page 5. The first approach involved using antibodies -- raised to cell surface proteins and selected based on their ability to block adhesion of cells to extracellular matrix proteins -- for affinity purification of an integrin, followed by aminoterminal sequencing of the isolated integrin protein and cDNA library screening. In the second approach, the antibodies were used for the screening of a cDNA expression library. Examples of integrin α chains that were cloned by these antibody-based approaches include α1, α2, and α3. The third approach involved PCR amplification of integrin α chain cDNAs using degenerate primers designed based on regions of homology between already known integrin α chains. Examples of integrin α chains that were cloned by this approach include α7 and α9.

These were the three approaches that one of ordinary skill in the art at the time of the invention would have taken in an attempt to clone integrin αmt. See Gullberg declaration, page 4; Johansson declaration, pages 5. In each of these approaches the same type of cells in which the integrin subunit had been identified would have served as the source material. Thus, for purposes of cloning integrin subunit αmt the source material would have been human myotube cells, and in particular G6 myotube cells. See Gullberg declaration, pages 5-6; Johansson declaration, pages 5-6. Because of the known complexity of the integrin family one of ordinary skill in the art would have been very reluctant to change the source material. A change of source material would

have been expected to dramatically reduce the expectation of success. See Gullberg declaration, pages 8-9; Johansson declaration, pages 5-6.

The inventor did indeed try to clone integrin αmt by each of the three approaches, but he failed. See Gullberg declaration, page 6; Johansson declaration, page 5. Because of the complexity of the integrin family described above one of ordinary skill in the art would have with very high probability isolated cDNAs of known integrin α chains or of alternative splice variants thereof, rather than of any new integrin α chain. This is exactly what happened in the inventor's attempts to clone integrin αmt by the above approaches. He isolated several different known integrin α chains, including at least integrin α1, α3, α4, α5, α6, α7, and αv, from G6 myotubes, but failed to isolate integrin αmt, the target of his efforts. Gullberg et al, page 58, paragraph 3, and page 59, paragraph 2. Integrin α7 which is expressed in G6 myotubes is a prime example of the complicated expression pattern of alternatively spliced products derived from the same gene.

The low expectation of success was reflected in the fact that no new integrin α chain was isolated for several years (from 1996 to 1998) despite the high motivation among investigators in the field and despite the availability of improved knowledge and reagents that would have facilitated the search for such new molecules. See Gullberg declaration, pages 7-8; Johansson declaration, pages 2-3. Because of this failure to isolate additional integrin subunits for several years it was then generally assumed in the integrin field that all α chains had been identified. See Gullberg declaration, pages 7-8; Johansson declaration, pages 2-3. In fact, a general skepticism towards any report of a newly identified integrin protein had developed at the time of the invention, in

particular after several publications had reported the cloning of "integrin-like" proteins which were not considered true integrin proteins. See Johansson declaration, pages 4-5.

In conclusion, one of ordinary skill in the art at the time of the invention certainly did not have a reasonable expectation of success.

That the expectation of success was exceedingly low was born out by the fact that the Applicant tried, but ultimately failed, to clone integrin αmt from G6 cells, using the most promising methods available at the time. See Gullberg declaration, pages 4-6. Similarly, other laboratories which had reported evidence of an unknown integrin α chain resembling integrin αmt or integrin α11 also failed to isolate the novel integrin subunit, even though the researchers involved were highly skilled in the field of integrin research. See Gullberg declaration, pages 6-7. Finally, the exceedingly low expectation of success was reflected by the fact that the Velling et al report of the cloning of integrin α11 came as a surprise to the field, despite the previous report by Gullberg et al postulating a novel integrin α subunit. See Johansson declaration, page 6.

In light of all of the above, Applicant submits that claims 156-162 are not obvious over Gullberg et al in view of Alberts et al (1989) and US patent No. 6,046,316. Applicant thus respectfully requests that the rejection of claims 156-162 under 35 U.S.C. § 103(a) be withdrawn.

Conclusion

As Applicants have addressed all of the Examiner's rejections and demonstrated that the now pending claims are patentable over the art of record, Applicants respectfully request entry and timely allowance of the pending claims. If the Examiner believes a telephone conference would be useful in resolving any outstanding issues, the Examiner is invited to call the undersigned at (202) 408-4173. Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
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Dated: January 17, 2008

By: _____
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Isolation, Cloning, and Sequence Analysis of the Integrin Subunit α10, a β1-associated Collagen Binding Integrin Expressed on Chondrocytes*

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We have found that chondrocytes express a novel collagen type II-binding integrin, a new member of the β1-integrin family. The integrin α subunit, which has a M_r of 160 kDa reduced, was isolated from bovine chondrocytes by collagen type II affinity purification. The human homologue was obtained by screening a human chondrocyte library with a bovine cDNA probe. Cloning and cDNA sequence analysis of the human integrin α subunit designated α10 show that it shares the general structure of other integrin α subunits. The predicted amino acid sequence consists of a 1167-amino acid mature protein, including a signal peptide (22 amino acids), a long extracellular domain (1098 amino acids), a transmembrane domain (25 amino acids), and a short cytoplasmic domain (22 amino acids). The extracellular part contains a 7-fold repeated sequence, an I-domain (199 amino acids) and three putative divalent cation-binding sites. The deduced amino acid sequence of α10 is 35% identical to the integrin subunit α2 and 37% identical to the integrin subunit α1. Northern blot analysis shows a single mRNA of 5.4 kilobases in chondrocytes. A peptide antibody against the predicted sequence of the cytoplasmic domain of α10 immunoprecipitated two proteins with masses of 125 and 160 kDa from chondrocyte lysates under reducing conditions. The peptide antibody specifically stained chondrocytes in tissue sections of human articular cartilage, showing that α10β1 is expressed in cartilage tissue.

The integrins are a large family of transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions (1–5). All known members of this superfamily are noncovalently associated heterodimers composed of an α and a β subunit. At present, 8 β-(β1–β8) (See Ref. 6 and references therein) and 16 α subunits (α1–α9, αv, αM, αL, αX, αIIb, αE, and αD) have been characterized (6–21), and these subunits associate to generate more than 20 different integrins. The β1 subunit has been shown to associate with 10 different α subunits, α1–α9 and αv and to mediate interactions with extracellular matrix proteins such as collagens, laminins, and fibronectin. The major collagen binding integrins are α1β1 and α2β1 (22–25). The integrins α3β1 and α9β1 have also been reported to interact with collagen (26, 27), although this interaction is not well understood (28). The extracellular N-terminal regions of the α and β integrin subunits are important in the binding of ligands (29, 30). The N-terminal region of the α subunits is composed of a 7-fold repeated sequence (12, 31) containing FG and GAP consensus sequences. The repeats are predicted to fold into a β-propeller domain (32), with the last three or four repeats containing putative divalent cation binding sites. The α-integrin subunits α1, α2, αD, αE, αL, αM, and αX contain an ~200 amino acid inserted domain, the I-domain (A-domain), that shows similarity to sequences in von Willebrand factor, cartilage matrix protein, and complement factors C2 and B (33, 34). The I-domain is localized between the second and third FG-GAP repeats; it contains a metal ion-dependent adhesion site (MIDAS), and it is involved in binding of ligands (35–38).

Chondrocytes, the only type of cells in cartilage, express a number of different integrins including α1β1, α2β1, α3β1, α5β1, α6β1, αvβ3, and αvβ5 (39–41). We have shown that α1β1 and α2β1 mediate chondrocyte interactions with collagen type II (25), which is one of the major components in cartilage. We have also shown that α2β1 is a receptor for the cartilage matrix protein chondroadherin (42). In the present study we have isolated a novel collagen type II binding integrin, α10β1, from bovine articular chondrocytes. Cloning and sequence analysis of the human homologue is described, and expression of α10 on chondrocytes is examined.

MATERIALS AND METHODS

Antibodies—A polyclonal antiserum was generated against the α10 cytoplasmic domain peptide CKKIPEEEKREEKLE. Peptide synthesis and conjugation to keyhole limpet hemocyanin, injection of rabbits and affinity purification were performed by Innovagen AB (Lund, Sweden). Monoclonal antibodies against human integrin subunit β1 (P4C10), α2 (P1E6), and α3 (P1B5) (unpurified ascites fluid) were from Life Technology Inc. The monoclonal antibody against human integrin subunit α1 (TS2/7; hybridoma supernatant) was a kind gift from Timothy Springer, Boston Blood Center, Boston, MA (43). Polyclonal antibody (serum) against the rat β1-integrin subunit was kindly provided by Staffan Johansson, Uppsala, Sweden (44). Polyclonal antibodies (serum) against human integrin subunits α2 (AB1936), α3 (AB1920), and polyclonal antibody (serum) against rat integrin subunits α1 (AB1934) were from Chemicon International Inc. (Temecula, CA). Polyclonal antibodies against the integrin subunit α9 (affinity-purified IgG) were a kind gift from Dean Sheppard, University of California San Francisco Lung Biology Center, San Fransisco, CA (6).

Cell Isolation and Culture—Bovine chondrocytes were isolated by digestion of articular cartilage from 4–6-month-old calves with collagenase (CLS1; Worthington Biochemical Corp., Lakewood, NJ) as described elsewhere (45). Briefly, cartilage slices were digested by colla-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF074015.

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genase in Earle's balanced salt solution (Life Technologies, Inc.) for 15–16 h at 37 °C. The tissue digest was filtered through a 100-μm nylon filter, and the isolated cells were then washed three times in Dulbecco's modified phosphate-buffered saline (PBS),[1] Life Technologies, Inc.). Human chondrocytes from articular cartilage were isolated by digestion with Pronase (Calbiochem) for 1 h followed by collagenase (Boehringer Mannheim) for 15–18 h, as described by Häuselmann *et al.* (46). The cells were filtered and washed as described above. Human chondrocytes were cultured in Dulbecco's minimum essential medium and F-12 (1:1) supplemented with 10% fetal calf serum, 25 μg/ml ascorbic acid, 50 IU of penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). To harvest cells, the culture dish was washed three times with Ca^{2+}/Mg^{2+}-free PBS, and the cells were incubated with 0.5% trypsin and 1 mM EDTA (Life Technologies, Inc.) in Ca^{2+}/Mg^{2+}-free PBS for 5 min. Detached cells were suspended in medium containing 10% fetal calf serum or in PBS containing 1 mg/ml trypsin inhibitor (Sigma) and then washed in PBS.

Coupling of Affinity Columns—Collagen type II isolated from nasal cartilage by pepsin digestion (47) was coupled to CNBr-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the published procedure (25). A control column was produced by treating CNBr-Sepharose 4B in a similar manner but in the absence of protein. Bovine fibronectin (Sigma) was coupled to CNBr-Sepharose 4B according to instructions from the manufacturer. After blocking, the fibronectin-Sepharose was washed three times with PBS.

Affinity Purification and Immunoprecipitation of Chondrocyte Membrane Proteins—Human chondrocyte cell surface proteins were ^{125}I-labeled and affinity-purified on collagen type II-Sepharose according to the published procedure (25). Cell lysates or affinity-purified samples were immunoprecipitated as described earlier (42). The following antibodies were used in immunoprecipitation experiments: monoclonal antibodies against the human integrin subunits β1,α1,α2, or α3 (unpurified ascites fluid, dilution 1/100), polyclonal antibody against the rat integrin subunit β1 (purified IgG, 50–100 μg/ml), polyclonal peptide antibodies against the integrin subunits α1, α2, α3, and α10 (serum, dilution 1/100). The immunoprecipitated proteins were separated by 4–12% SDS-PAGE and visualized by image analysis using the BioImaging Analyzer Bas2000 (Fuji Photo Film Co., Tokyo, Japan).

Western Blot—Human chondrocyte membrane proteins immunoprecipitated with polyclonal antibodies against α10 (10 μg/ml affinity-purified IgG) or β1 (100 μg/ml IgG) were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane essentially as described by Towbin *et al.* (48). The membrane was blocked with 3% dried milk in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.2% Tween (blocking buffer) and then incubated with the β1 antibody (20 μg/ml) in blocking buffer containing 1% dried milk. The β1 subunit was detected after incubation with a secondary antibody conjugated with horseradish by chemiluminescence using the ECL system (Amersham Pharmacia Biotech).

Affinity Purification of the Integrin Subunit α10 on Collagen Type II-Sepharose—Freshly isolated bovine chondrocytes (2500 × 10^6) were lysed in 6 ml of 1% Triton X-100, 100 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4, for 1 h on ice. The lysate was centrifuged for 30 min at 10,000 rpm, and the pellet was discarded. Collagen type II-Sepharose (4 ml) and the fibronectin-Sepharose (2 ml) were equilibrated with at least 20 volumes of 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4 (equilibration buffer). The entire cell lysate was passed over the fibonectin-Sepharose twice, and the flow through was then incubated with the collagen-Sepharose end over end for 3 h. The columns were washed (15 gel volumes) with the equilibration buffer containing 75 mM NaCl, and bound proteins were eluted with 20 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl, pH 7.4.

Isolation of Internal Peptides by In-gel Digestion and Peptide Sequencing—Affinity-purified proteins were concentrated by precipitation using the methanol/chloroform protocol (49). After reduction/alkylation with dithiothreitol/iodoacetamide (50), the precipitated proteins were subjected to SDS-PAGE on a 4–12% polyacrylamide gel, and protein bands were visualized by Coomassie staining. The 160-kDa protein band was excised from the gel and prepared for in-gel digestion (51). Briefly, the gel slice was washed extensively to remove SDS and the dye, and after complete drying, protease was forced into the gel by rehydration with a solution of modified trypsin (Promega, Madison, WI) in 0.2 M NH_4HCO_3 buffer. After an overnight incubation, peptides were extracted and then isolated by narrow bore reversed phase liquid chromatography on a μRPC C2/C18 stainless steel 2.1/10 column operated in a SMART System (Amersham). Several peptides were analyzed by Edman degradation in a Perkin-Elmer Applied Biosystem Model 476 sequencer operated according to the manufacturer's instructions.

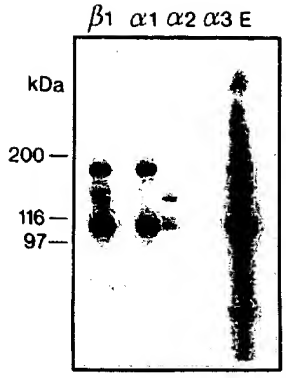

FIG. 1. **Affinity purification and immunoprecipitation of collagen type II-binding integrins from Triton X-100 lysate of ^{125}I-labeled human chondrocytes.** The *lanes* show immunoprecipitation of integrins using monoclonal antibodies against the integrin subunits β1(P4C10), α1(TS2/7), α2(P1E6),and α3(P1B5). The proteins eluted by EDTA from the collagen type II-Sepharose are shown in *lane E*. The proteins were separated by SDS-PAGE (4–12%) under nonreducing conditions and visualized using phosphoimaging.>

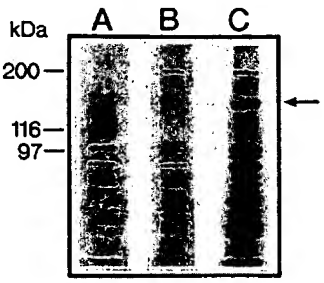

FIG. 2. **Affinity purification of the α10 integrin subunit on collagen type II-Sepharose.** A Triton X-100 lysate of bovine chondrocytes (2.5 × 10^9 cells) was applied to a fibronectin-Sepharose precolumn followed by a collagen type II-Sepharose column. The *lanes* show EDTA-eluted proteins from the fibronectin-Sepharose (*A*), flow-through from the collagen type II-Sepharose column (*B*), and EDTA-eluted proteins from the collagen type II-Sepharose (*C*). The eluted proteins were precipitated by methanol/chloroform, separated by SDS-PAGE (4–12%) under reducing conditions, and stained with Coomassie Blue. The 160-kDa protein with affinity for collagen type II is indicated with an *arrow*.

mRNA Purification and cDNA Synthesis—mRNA from bovine or human chondrocytes were isolated using a QuickPrep® Micro mRNA purification kit (Pharmacia). cDNA was synthesized at 42 °C for 1 h using the Superscript™ II RNase H⁻ Reverse Transcriptase cDNA Synthesis system (Life Technologies, Inc.) random DNA hexamers and oligo(dT) (Promega, Madison, WI).

PCR Amplification—PCR reactions were performed in 50-μl reaction volumes and contained 1 × *Taq* polymerase buffer (Life Technologies, Inc.), 1.5 mM $MgCl_2$, 1 μM of each primer, 0.025 units/μl *Taq* polymerase, 1 μl of DNA template (bovine chondrocyte cDNA), and 0.1 mM each of dATP, dGTP, dCTP, and dTTP (Boehringer Mannheim). PCR samples were heated to 94 °C for 5 min in a thermocycler and then subjected to 35 cycles consisting of 30 s at 94 °C (denaturation), 30 s at 48 or 52 °C (annealing) and 3 min at 72 °C (extension). The PCR products were re-amplified using 1 μl of each product for an additional 35 cycles. Amplified DNA was analyzed by 1% agarose gel electrophoresis. Small DNA fragments were analyzed using 4% MethaPhore™-agarose (FMC BioProducts, Rockland, ME).

The degenerate primers GAY AAY ACI GCI CAR AC (DNTAQT, forward) and TIA TIS WRT GRT GIG GYT (EPHHSI, reverse) were used in PCR to amplify the nucleotide sequence corresponding to the bovine peptide 1 (Table I). A 900 base pair PCR fragment was then amplified from bovine cDNA using an internal specific primer TCA GCC TAC AT-

[1] The abbreviations used are: PBS, phoshate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE (Race), rapid amplification of the cDNA end.

T CAG TAT (SAYIQY, forward) corresponding to the cloned nucleotide sequence of peptide 1 together with the degenerate primer ICK RTC-CCA RTG ICC IGG (PGHWDR, reverse) corresponding to the bovine peptide 2 (Table I). Mixed bases were used in positions that were 2-fold degenerate, and inosines were used in positions that were 3- or 4-fold degenerate.

To obtain cDNA that encoded the 5′ end of α10, we designed the primer AAC TCG TCT TCC AGT GCC ATT CGT GGG (reverse; residues 1254–1280 in α10 cDNA) and used it for rapid amplification of the cDNA 5′ end (RACE) as described in the Marathon™ cDNA amplification kit (CLONTECH INC., Palo Alto, CA).

Cloning and Sequencing of cDNA—PCR fragments were isolated and

TABLE I

Amino acid sequences of peptides from bovine α10-integrin

Peptides were isolated by in-gel digestion with trypsin and sequenced by Edman degradation.

Peptide	Amino acid sequence
1	DNTAQTSAYIQYEPHHSI
2	GPGHWDR
3	AAFDGSGQR
4	FAMGALPD
5	FTASLDEWTTAAR
6	VDASFRPQGXLAP

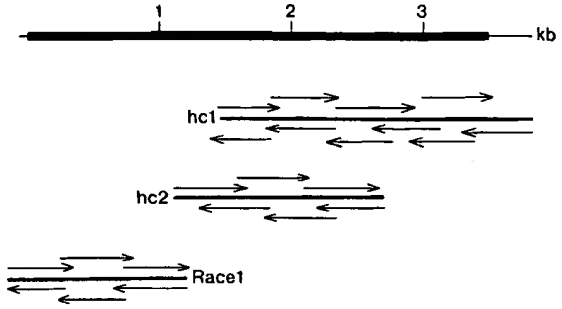

FIG. 3. **Schematic map of the sequencing strategy.** The overlapping α10 clones hc1 and hc2 were obtained by screening a human articular chondrocyte library with a bovine α10 probe. The Race1 clone was obtained from human chondrocyte cDNA using the RACE technique. *Arrows* indicate the direction and extent of nucleotide sequencing. *kb*, kilobases.

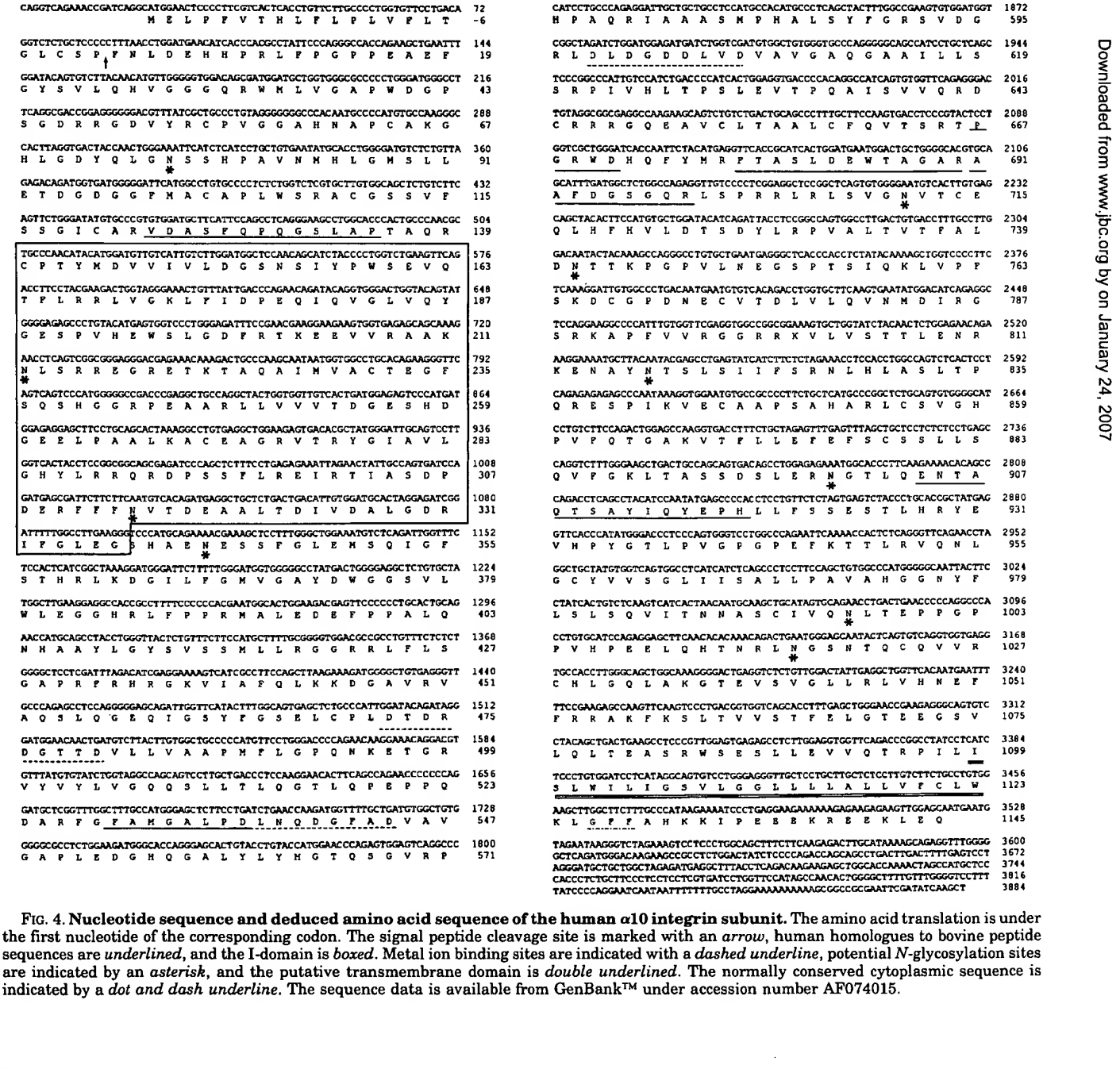

FIG. 4. **Nucleotide sequence and deduced amino acid sequence of the human α10 integrin subunit.** The amino acid translation is under the first nucleotide of the corresponding codon. The signal peptide cleavage site is marked with an *arrow*, human homologues to bovine peptide sequences are *underlined*, and the I-domain is *boxed*. Metal ion binding sites are indicated with a *dashed underline*, potential *N*-glycosylation sites are indicated by an *asterisk*, and the putative transmembrane domain is *double underlined*. The normally conserved cytoplasmic sequence is indicated by a *dot and dash underline*. The sequence data is available from GenBank™ under accession number AF074015.

purified from agarose gels using Jet Sorb DNA extraction kit (Genomed Inc. Research Triangle Park, CA). Purified fragments were then cloned with the pCR Script™ Sk(+) kit (Stratagene, La Jolla, CA). Selected plasmids were purified from liquid cultures using QIAGEN plasmid midi preparation kit (QIAGEN Inc. Valencia, CA) and sequenced by ABI 373A sequencer using ABI Prism™Dye Terminator Cycle Sequencing Core kit (Perkin-Elmer) together with T3, T7, and internal specific primers.

Library Screening—The cloned 900-base pair PCR fragment corresponding to bovine α10-integrin was digoxigenin-labeled according to the DIG DNA labeling kit (Boehringer Mannheim) and used as a probe for screening of a human articular chondrocyte λZapII cDNA library (provided by Michael Bayliss, The Royal Veterinary Basic Sciences, London, UK) (52). Positive clones containing the pBluescript SK^+ plasmid with the cDNA insert were rescued from the ZAP vector by *in vivo* excision as described in the ZAP-cDNA® synthesis kit (Stratagene). Selected plasmids were purified and sequenced as described earlier using T3, T7, and internal specific primers.

Northern Blot Analysis—Bovine chondrocyte mRNA was purified using a QuickPrep®Micro mRNA purification kit (Amersham), separated on a 1% agarose formaldehyde gel, transferred to nylon membranes, and immobilized by UV cross-linking. cDNA probes were ^{32}P-labeled with Random Primed DNA labeling kit (Boehringer Mannheim). Filters were prehybridized for 2–4 h at 42 °C in 5× SSE (20 × SSC, 3M NaCl, 0.3 M trisodium citrate·$2H_2O$, pH adjusted to 7.0 with 1 M HCl), 5× Denhardt's solution, 0.1% SDS, 50 μg/ml salmon sperm DNA, and 50% formamide and then hybridized overnight at 42 °C with the same solution containing the specific probe (0.5–1 × 10^6 cpm/ml). Specifically bound cDNA probes were analyzed using the phosphoimaging system (Fuji). Filters were stripped by washing in 0.1% SDS for 1 h at 80 °C before reprobing. The α10-integrin cDNA probe was isolated from the race1-containing plasmid using the restriction enzymes *Bam*HI (Life Technologies, Inc.) and *Nco*I (Boehringer Mannheim). The rat β1-integrin cDNA probe was a kind gift from Staffan Johansson, Uppsala, Sweden (25).

Tissue Staining—Human cartilage from the trochlear groove, obtained during surgery, was provided by Anders Lindahl, Sahlgrenska University Hospital, Gothenburg, Sweden. Frozen sections of cartilage tissue were fixed in acetone at −18 °C for 5 min, washed in PBS, and then treated with 2 mg/ml hyaluronidase (Sigma) in PBS, pH 5.0, for 15 min at 37 °C. After washing with PBS, sections were blocked for 15 min at room temperature in 0.1% H_2O_2 in PBS to remove endogenous peroxidase activity. Sections were then washed in PBS, blocked with 0.5% casein and 0.05% thimerosal in PBS (blocking buffer) for 15 min at room temperature, and then incubated overnight at 4 °C with the affinity-purified antibodies against the integrin subunits α9 or α10 (5 μg/ml in blocking buffer). For control, the α10 antibody was preincubated with the α10 peptide (0.1 mg/ml) for 30 min at 4 °C. After washing in PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Inc; diluted 1:200 in blocking buffer) at room temperature for 60 min. Washed sections were then incubated with VECTASTAIN® ABC reagent (Vector Laboratories, Inc. Burlingame, CA) for 1 h at room temperature and washed, and the color was developed using 1 mg/ml diaminobenzidine, 0.02% H_2O_2 and 0.1 M Tris-HCl, pH 7.2. Sections were rinsed in water for 5 min followed by 75, 95, and 99.5% ethanol for 5 min each and then three times in xylene for 3 min at room temperature. Samples were mounted in Pertex (Histolab Products AB, Gothenburg, Sweden) and examined by light microscopy.

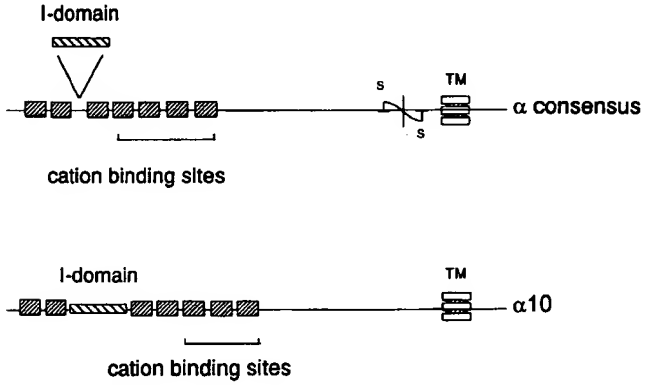

FIG. 5. **Diagrammatic comparison of α10 with the general structure of integrin α subunits.** The conserved repeats 1–7 are indicated with *boxes*. An I-domain is found in some α-integrin subunits. Other α integrin subunits are posttranslationally cleaved near the transmembrane domain (*TM*). The integrin subunit α10 contains the seven conserved repeats and an I-domain located between repeat two and three.

RESULTS

Identification and Isolation of the Chondrocyte α10 Integrin Subunit—Affinity purification of ^{125}I-labeled membrane proteins from human chondrocytes on collagen type II-Sepharose followed by immunoprecipitation showed that these cells, in addition to α1β1 and α2β1, express an unidentified β1-related α subunit (Fig. 1). This integrin subunit had an apparent molecular mass of approximately 160 kDa under reducing condition and was slightly larger than the α2 integrin subunit. This finding is in agreement with a previous study from our group showing that bovine chondrocytes also express an unidentified collagen binding β1-associated α subunit of similar molecular mass (25). To isolate this protein, we affinity-purified collagen type II-binding proteins from bovine chondrocytes. The chondrocyte lysate was first applied to a fibronectin-Sepharose precolumn, and the flow-through was then applied to a collagen type II-Sepharose column. As shown in Fig. 2, a number of proteins were eluted from the affinity columns. A protein with molecular mass of approximately 160 kDa was specifically eluted with EDTA from the collagen column but not from the fibronectin column. The molecular mass of this protein corresponded with the molecular mass of the unidentified β1-related integrin subunit (Fig. 1). The 160-kDa protein band was excised from the SDS-PAGE gel and digested with trypsin, and several of the isolated peptides were analyzed. Table I shows the amino acid sequence of six individual peptides.

Cloning and Sequencing of the Human Integrin α-Subunit Homologue—The nucleotide sequence corresponding to peptide 1 (Table I) was obtained by PCR amplification, cloning, and sequencing of bovine cDNA. From this nucleotide sequence an exact primer was designed and applied in PCR amplification with degenerate primers corresponding to peptides 2–6 (Table I). Primers corresponding to peptides 1 and 2 amplified a 900-base pair PCR fragment from bovine cDNA that was cloned, sequenced, and used for screening of a human articular chondrocyte λZapII cDNA library to obtain the human integrin α-subunit homologue. Two overlapping clones, hc1 and hc2 (Fig. 3), were isolated, subcloned, and sequenced. These clones

TABLE II
Comparison of the cytoplasmic tails of I-domain-containing integrin α subunits
The underlined sequence in α10 represents the peptide that was used for antibody production.

Peptide	Amino acid sequence
α1	KIGFFKRPLKKKMEK
α2	KLGFFKRKYEKMTKNPDEIDETTELSS
α10	KLGFFAHKKIPEEEKREEKLEQ
αM	KLGFFKRQYKDMMSEGGPPGAEPQ
αX	KVGFFKRQYKEMMEEANGQIAPENGTQTPSPPSEKPSEK
αL	KVGFFKRNLKEKMEAGRGVPNGIPAEDSQLASGQEAGDPGCLKPLHEKDSESGGGKD
αE	KCGFFKRKYQQLNLESIRKAQLKSENLLEEEN

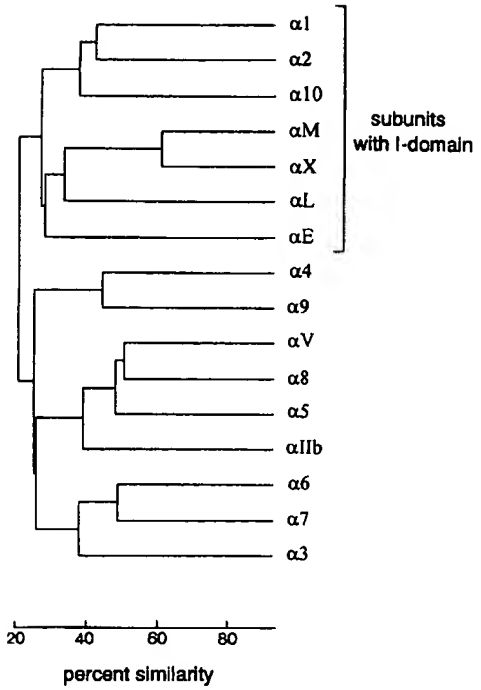

FIG. 6. **Sequence similarity between integrin α subunits.** The similarity tree was constructed using the GCG software and the program "Pileup." Percent identities were calculated using the Jotun Hein algorithm provided in the Lasergene DNASTAR software. The similarity tree indicates three different subfamilies of α-integrin subunits, one subfamily that contains I-domains (α1, α2, αM, αX, αL, and α10), one subfamily that is cleaved (α3, α5, α6, α7, α8, αv, and αIIb), and one subfamily that neither contains I-domains nor is cleaved (α4, α9).

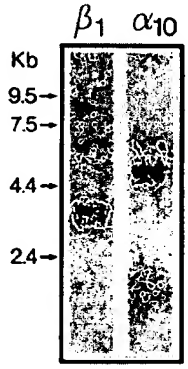

FIG. 7. **Northern blot analysis of α10 and β1 mRNA.** Bovine chondrocyte mRNA was hybridized with ^{32}P-labeled cDNA probes corresponding to the integrin subunits α10 or β1. The α10 probe hybridized to an mRNA of 5.4 kilobases, and the β1-probe hybridized to an mRNA of 3.5 kilobases on the same filter.

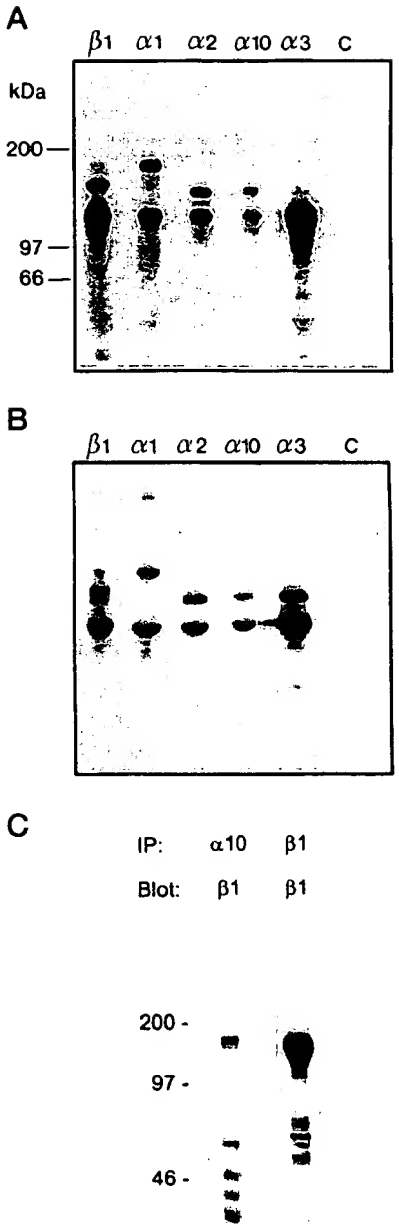

FIG. 8. **Immunoprecipitation of the α10 integrin subunit from human chondrocytes.** Triton X-100 lysates of ^{125}I-labeled human chondrocytes were immunoprecipitated with polyclonal antibodies against the integrin subunits β1, α1, α2, α3, or α10. The immunoprecipitated proteins were separated by SDS-PAGE (4–12%) under reducing (*a*) and nonreducing conditions (*b*) and visualized using a phosphoimager (*c*). Triton X-100 lysates of human chondrocytes immunoprecipitated with α10 or β1 were separated by SDS-PAGE (8%) under nonreducing conditions and analyzed by Western blot using the polyclonal β1 antibody and chemiluminescent detection. *C*, control.

contained ⅔ of the nucleotide sequence, including the 3′ end of the cDNA. A third clone (*Race1*; Fig. 3), which contained the 5′end of the α10 cDNA, was obtained using the RACE technique. From these three overlapping clones of α10 cDNA, 3884 nucleotides were sequenced (Fig. 4). The sequence contains a 3504-nucleotide open reading frame that is predicted to encode a 1167 amino acid mature protein. The predicted sequence included a signal peptide (22 amino acids), a long extracellular domain (1098 amino acids), a transmembrane domain (25 amino acids), and a short cytoplasmic domain (22 amino acids). Sequence analysis of the 160-kDa protein sequence showed that it was a member of the integrin α-subunit family, and the subunit was named α10.

Comparison of α10 Integrin Subunit with Other α Subunits—Analysis of α10 with known α subunits showed that its structure follows the conserved pattern of integrin α subunits (Fig. 5). The extracellular domain contains a 7-fold repeated sequence including FG and GAP consensus sequences, three putative divalent cation binding sites (D*X*D/N*X*D/N*XXX*D), and an I domain of 199 amino acids. The protein contains 10 potential *N*-linked glycosylation sites (N*X*(T/S)). The calculated molecular mass is 153 kDa if carbohydrate chains with an average molecular weight of 2.5 kDa are assumed to attach to all 10 putative glycosylation sites. This is in agreement with the molecular mass of α10 as judged by SDS-PAGE where the molecular mass was estimated to approximately 160 kDa.

In contrast to most α-integrin subunits, the cytoplasmic do-

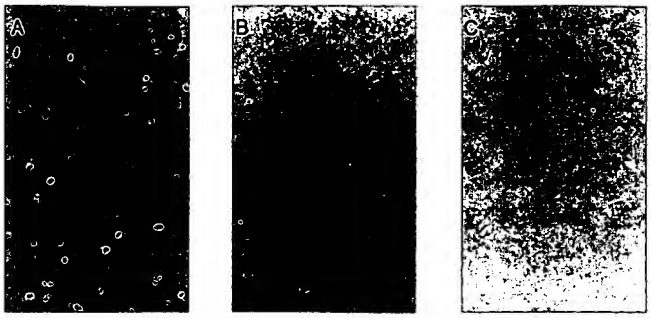

FIG. 9. **Immunostaining of human articular cartilage.** An antibody raised against the cytoplasmic domain of α10 (see Table II) stained the chondrocytes in tissue sections of human articular cartilage (*A*). The staining was depleted when the antibody was preincubated with the α10 peptide (*B*). A control antibody recognizing the α9 integrin subunit did not bind to the chondrocytes (*C*).

main of α10 does not contain the conserved sequence KXGFF(R/K)R (Table II). Instead, the predicted amino acid sequence is KLGFFAH. The deduced amino acid sequence of α10 showed the highest identity to the collagen-binding integrin subunits α1 (37%) and α2 (35%). The similarity of integrin α subunits are shown in Fig. 6.

Expression of the α10 Integrin Subunit on Chondrocytes—Northern blot analysis of mRNA from bovine chondrocytes showed that a human α10 cDNA probe hybridized with a single mRNA of approximately 5.4 kilobases (Fig. 7). As a comparison, a cDNA probe corresponding to the integrin subunit β1 was used. This cDNA probe hybridized a mRNA band of approximately 3.5 kilobases on the same filter. Translation of the α10 nucleotide sequence revealed an open reading frame of 3504 nucleotides (Fig. 4), which indicates that around 2000 nucleotides in the mRNA is not translated.

To study expression of α10 at the protein level, ^{125}I-labeled membrane proteins from human chondrocytes were immunoprecipitated with polyclonal antibodies against the integrin subunits β1, α1, α2, α3, and α10 (Fig. 8). A polyclonal peptide antibody raised against the cytoplasmic domain of α10 precipitated two protein bands with molecular masses of approximately 160 and 125 kDa under reducing conditions. The α10-associated β-chain migrated as the β1 integrin subunit both under reducing and nonreducing conditions (Figs. 8, *a* and *b*). To verify that the α10-associated β-chain indeed is β1, chondrocyte lysates were immunoprecipitated with antibodies against α10 or β1 followed by Western blot using antibodies against the β1 subunit (Fig. 8*c*). These results clearly demonstrated that α10 is a member of the β1-integrin family.

Expression of α10 in cartilage was examined by immunostaining of human articular cartilage from the trochlear groove with the polyclonal α10 antibody. As shown in Fig. 9, this antibody specifically stained the chondrocytes in the cartilage tissue sections. The staining was completely abolished when the antibody was preincubated with the α10 peptide. A control antibody against the α9 integrin subunit did not stain chondrocytes in the tissue sections (Fig. 9).

DISCUSSION

The present study demonstrated that human chondrocytes express a novel, collagen type II-binding integrin in the β1 family. We have, in an earlier study, presented some evidence for that bovine chondrocytes and human chondrosarcoma cells also express this integrin (25). Because bovine chondrocytes are readily available in large amounts, we used these cells in the isolation of the integrin subunit α10. As shown in Fig. 2, several proteins were eluted from the columns in the affinity purification experiments. It was difficult to interpret the protein pattern in the eluate because typical integrin bands were not clearly distinguished on the SDS-PAGE gel. This may be explained by partial protein degradation, although a mixture of protease inhibitors were included in the lysate buffer. Based upon the finding that the β1 antibody immunoprecipitated an unknown collagen-binding integrin α subunit with a molecular mass of 160 kDa (Fig. 1), a protein with similar molecular mass that was specifically eluted with EDTA from the collagen type II column was excised from the gel and used for peptide sequencing. This 160-kDa protein was not eluted from the fibronectin-Sepharose, indicating that fibronectin is not a ligand for α10β1. However, this will be investigated in cell adhesion experiments using cells transfected with the α10 subunit.

The immunoprecipitation experiments showed that α2 and α10 integrin subunit have similar molecular masses under reducing conditions (Fig. 1). To avoid contamination of α2, the 160-kDa protein was excised from the SDS-PAGE gel as a very narrow band. This was apparently successful since human homologues to all six bovine peptides (Table I) that were isolated from the 160-kDa protein were found in the predicted amino acid sequence of human α10 subunit (Fig. 4).

The deduced amino acid sequence of α10 was found to share the general structure of the integrin α subunits described in previously published reports (6–21). The large extracellular N-terminal part of α10 contains a 7-fold repeated sequence that was recently predicted to fold into a β-propeller domain (32). The integrin subunit α10 contains three putative divalent cation binding sites (DXD/NXD/NXXXD) (53), a single spanning transmembrane domain, and a short cytoplasmic domain. In contrast to most α-integrin subunits, the cytoplasmic domain of α10 does not contain the conserved sequence KXGFF(R/K)R. The predicted amino acid sequence in α10 is KLGFFAH. Several reports indicate that the integrin cytoplasmic domains are crucial in signal transduction (54) and that membrane-proximal regions of both α- and β-integrin cytoplasmic domains are involved in modulating conformation and affinity state of integrins (55–57). It is suggested that the GFFKR motif in α-chains are important for association of integrin subunits and for transport of the integrin to the plasma membrane (58). The KXGFFKR domain has been shown to interact with the intracellular protein calreticulin (59), and interestingly, calreticulin-null embryonic stem cells are deficient in integrin-mediated cell adhesion (60). It is, in this context, tempting to speculate that the sequence KLGFFAH in α10 may have a key function in regulating the affinity between α10β1 and collagen.

Integrin α subunits are known to share an overall identity of 20–40% (61). Sequence analysis showed that the α10 subunit is most closely related to the I domain-containing α subunits (Fig. 6) with the highest identity to α1 (37%) and α2 (35%). The integrins α1β1 and α2β1 are known receptors for both collagens and laminins (24, 62, 63), and we have also recently demonstrated that α2β1 interacts with the cartilage matrix protein chondroadherin (42). Since α10β1 was isolated on a collagen type II-Sepharose, we know that collagen type II is a ligand for α10β1. We have also shown by affinity purification experiments that α10β1 interacts with collagen type I (data not shown), but it remains to be seen whether laminin or chondroadherin are also ligands for this integrin.

The peptide antibody that we raised against the cytoplasmic domain of α10 immunoprecipitated two proteins from human chondrocytes with molecular masses of approximately 125 and 160 kDa. The molecular mass of 160 kDa correlates with the unidentified β1-associated α subunit that was affinity-purified on collagen type II-Sepharose. The 125-kDa protein was in Western blot recognized by an antibody to the β1 subunit. This,

together with previous findings that α1β1 and α2β1 are present on isolated chondrocytes demonstrate that chondrocytes express at least three collagen-binding integrins in the β1 family (25). Further studies will answer the question whether these integrins have similar or different functions in cartilage.

Immunohistochemistry using the α10 antibody showed staining of the chondrocytes in tissue sections of human articular cartilage. The antibody staining was clearly specific because preincubation of the antibody with the α10 peptide completely abolished the staining. An antibody against the integrin subunit α9 did not stain the chondrocytes (6). This integrin is a receptor for tenascin C (64) and is not known to be present in cartilage.

Taken together, we have isolated and characterized a novel collagen type II-binding integrin designated α10β1. The α10 subunit was isolated from bovine chondrocytes, and the human homologue was cloned and sequenced. Antibodies against the α10-integrin subunit stained chondrocytes in tissue sections of articular cartilage, indicating that α10β1 indeed is expressed in cartilage. Further investigations including ligand interactions, tissue distribution, signal transduction, and knockout mutation will demonstrate the function of the integrin α10β1.

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The Laminin-binding Activity of the α7 Integrin Receptor Is Defined by Developmentally Regulated Splicing in the Extracellular Domain

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The expression pattern of the laminin-binding α7β1 integrin is developmentally regulated in skeletal, cardiac, and smooth muscle. The X1/X2 alternative splicing in the extracellular domain of α7 is found in the variable region between conserved α-chain homology repeat domains III and IV, a site implicated in ligand binding. To assess differences in X1/X2 isoform activity, we generated MCF-7 cell lines transfected with α7-X1/X2 cDNAs. Transfectants expressing the α7-X2 variant adhered rapidly to laminin 1, whereas those expressing α7-X1 failed to attach. That α7-X1 exists in an inactive state was established in assays using an activating β1 antibody that induced X1-dependent cell adhesion and spreading. Furthermore, the activation of α7-X1 was cell type specific, and when expressed in HT1080 cells, the integrin was converted into a fully functional receptor capable of promoting adhesion. Thus, the expression of the α7-X1/X2 integrin is a novel mechanism that regulates receptor affinity states in a cell-specific context and may modulate integrin-dependent events during muscle development and repair.

INTRODUCTION

Integrins are members of a large family of cell surface receptors that mediate adhesive interactions with extracellular matrix macromolecules (Hynes, 1992). Numerous studies have indicated that integrins are dynamically regulated, existing in a relatively nonfunctional state in which they are incompetent to bind ligand or in a fully active ligand bound form (Diamond and Springer, 1994; Humphries, 1996; Mould, 1996). Frequently, the ligand-binding regions of integrin subunits are cryptic and only become exposed when the subunit has been activated. A number of factors, originating from outside the cell or from the cell interior, can shift the inactive extracellular conformation of the integrin to an active one. For example, antibodies that stimulate integrin function have been shown to bind the extracellular domain of the β1 subunit (Takada and Puzon, 1993; Diamond and Springer, 1994; Humphries, 1996; Mould, 1996). These activating antibodies bind numerous epitopes, located primarily in the N-terminal region and cysteine-rich region of the β1 subunit, that either induce or stabilize the integrin complex in an active conformation that is competent to bind ligand (Arroyo *et al.*, 1992; Takada and Puzon, 1993; Bazzoni *et al.*, 1995). It is also well established that divalent cations can alter integrin-ligand interactions. For example, Mn^{2+} and Mg^{2+} can promote ligand binding, whereas Ca^{2+} acts as an inhibitor for a number of the integrin complexes (Humphries, 1996; Sanchez-Mateos *et al.*, 1996). Because integrin ligand recognition sites lie at or near the divalent cation binding regions, it has been proposed that divalent cations can directly induce a conformational change that exposes the ligand binding sites (Mould, 1996).

In contrast to external factors such as cations and stimulating antibodies, other effectors can activate integrin subunits intracellularly. For example, phorbol esters, by simulating the protein kinase C pathway, can increase the adhesiveness of several cell types through specific integrins (Shimizu *et al.*, 1990; Diamond and Springer, 1994). Finally, activation of cer-

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tain integrins is dependent on cell-type–specific regulation (Chan and Hemler, 1993) that may be due to posttranslational modifications, variations in the sequences of the α or β subunits, and/or the activation of different signal transduction molecules (Chan and Hemler, 1993; Hughes *et al.*, 1997).

Additional complexity of integrin regulation is exemplified by that fact that integrin subunits can be alternatively spliced. This alternative splicing has been shown to occur not only in a number of mammalian α and β subunits but also in *Drosophila* integrin receptors (Brown *et al.*, 1989; van Kuppevelt *et al.*, 1989; Suzuki and Naitoh, 1990; Cooper *et al.*, 1991; Hogervorst *et al.*, 1991; Tamura, *et al.*, 1991; Languino and Ruoslahti, 1992; Collo *et al.*, 1993; Song *et al.*, 1993; Ziober *et al.*, 1993; Djaffar *et al.*, 1994; Delwel *et al.*, 1995; Meredith *et al.*, 1995; Zhidkova *et al.*, 1995; Belkin *et al.*, 1996). Such splicing, which generally takes place in the cytoplasmic and the extracellular domains, can give rise to a number of subunit isoforms with diverse functional properties (Grinblat *et al.*, 1994; Fornaro *et al.*, 1995; Meredith *et al.*, 1995; Tennenbaum *et al.*, 1995; van der Flier *et al.*, 1995; Belkin, *et al.*, 1996; Roote and Zusman, 1996).

A laminin-binding integrin complex designated $\alpha7\beta1$ has recently been identified and shown to be expressed in skeletal, cardiac, and smooth muscle (Kramer *et al.*, 1991; Song *et al.* 1992; Collo *et al.*, 1993; Ziober *et al.*, 1993; Yao *et al.*, 1997). Investigating the function of this receptor has been complicated by the number of alternatively spliced isoforms of the $\alpha7$ subunit (Song *et al.* 1992; Collo *et al.*, 1993; Ziober *et al.*, 1993). In the cytoplasmic domain, two isoforms, A and B, are expressed in a developmentally specific manner in skeletal muscle. Although an $\alpha7$C variant has been reported in rat myoblasts, its role in development is unknown (Song *et al.*, 1993). Alternative splicing, in the extracellular domain of this subunit, also produces two muscle-specific developmentally regulated isoforms, designated X1 and X2 (Ziober, *et al.*, 1993). This extracellular alternative splicing occurs in the variable region between the III and IV homology repeat domains near the putative ligand-binding site, where it may define ligand specificity, affinity, or even ligand binding competence (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Mould, 1996). In this study we transfected $\alpha7$-X1 and $\alpha7$-X2 into MCF-7 cells to characterize the function of each isoform. $\alpha7$-X2 bound laminin readily, but $\alpha7$-X1 bound only when activated by the $\beta1$-activating monoclonal antibody (mAb)[1] TS2/16, indicating that alternative splicing regulates $\alpha7\beta1$ ligand binding competence. Furthermore, we show that activation of this isoform is regulated in a cell-specific manner.

[1] Abbreviations used: mAb, monoclonal antibody; OPG, octyl β-D-glycopyranoside.

MATERIALS AND METHODS

Cell Culture and Materials

The human breast carcinoma cell line MCF-7 was obtained from the American Type Culture Collection and maintained in DMEM H-16 with 10% fetal bovine serum. The human fibrosarcoma cell line HT1080 was obtained from American Type Culture Collection and maintained in DMEM plus 10% fetal bovine serum. Human plasma fibronectin was purchased from Collaborative Biomedical Products (Bedford, MA). Laminin 1 and the laminin E8 fragment were purified from mouse Engelbreth–Holm–Swarm tumor as described previously (Kramer *et al.*, 1991; Kramer, 1994). Human laminin 5 was kindly provided by Dr. Robert Burgeson (Cutaneous Biology Research Center, Boston, MA). Human type I collagen was obtained from Collagen Biomaterials (Palo Alto, CA).

Antibodies against integrin subunits included the rat anti-human $\beta1$ mAb A2B2 and the rat anti-human $\alpha5$ mAb B2G2, kindly provided by Dr. Caroline Damsky (University of California, San Francisco, CA); mouse anti-human $\alpha2$ mAb VM1, kindly provided by Dr. Vera Morhenn (SRI International, Menlo Park, CA); the $\beta1$-activating antibody TS2/16, kindly provided by Dr. Martin Hemler (Harvard Medical School, Boston, MA), and LM230, an anti-human αv mAb, kindly provided by Dr. Dean Sheppard (San Francisco General Hospital, San Francisco, CA). Anti-human $\alpha3$ mAb P1B5 was purchased from Life Technologies (Gaithersburg, MD); rat anti-human $\alpha6$ mAb GoH3 was purchased from AMAC (Westbrook, ME). The rabbit polyclonal antibody 1211 was prepared in this laboratory against peptide sequences specific to the $\alpha7$B cytoplasmic region (GTIQRSNWGNSQWEGSDAH; Yao *et al.*, 1996b). Rat anti-mouse $\alpha7$ mAbs CA5, CY4, and CY8 were generated in this laboratory as described previously (Yao *et al.*, 1996a). Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) and ECL kit were purchased from Amersham (Arlington Heights, IL).

Transfection

The construction of $\alpha7$-X2B cDNA has been described (Yao *et al.*, 1996b). Primers (5′ primer, 5′-CTCGACAGGAAGTGGACCAG-3′; 3′ primer, 5′-TGATCCCAAACATGGAATCAG-3′) that flank the X1/X2 splice site were used to amplify a 430-bp fragment from C2C12 myoblast mRNA by using reverse transcription-coupled polymerase chain reaction (PCR) and Vent polymerase (with proofreading activity, New England Biolabs, Beverly, MA). This fragment was digested with *Esp*I and *Apa*I and ligated into the corresponding sites of the $\alpha7$B cDNA. Positive clones containing the X1 insert were identified by PCR using primers specific for X1 and repeat region IV (5′ primer, 5′-GCCAGGGTGGAGCTCTG-3′; 3′ primer, 5′-CTATCCTTGCGCAGAATGAC-3′). The X1 PCR inserts were confirmed by double-stranded DNA sequencing.

Transfection of MCF-7 cells was performed by the calcium phosphate precipitation method (Mammalian Transfection kit, Stratagene, La Jolla, CA). MCF-7 cells at 30% confluency were transfected with 25–30 μg of DNA/10-cm plate. Cells were selected in growth medium containing 500 μg/ml G418. Individual clones or cell lines were isolated by using cloning rings or by fluorescence-activated cell sorting (FACS). Expression of $\alpha7$ in positive clones was verified by Western blot analysis using polyclonal antibody 1211 (Yao *et al.*, 1996b).

Western Blot

Transfected cell lines and parental cells were solubilized with SDS-solubilization buffer (50 mM Tris, pH 7.5, 0.5% Triton X-100, 1 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM *N*-ethylmaleimide). Equal amounts of protein were separated by

SDS-PAGE on 7.5% gels under nonreducing conditions, transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and then incubated with α7 polyclonal antibody 1211 followed by goat anti-rabbit IgG-HRP. Migration of the α7 subunit was determined by using an enhanced chemiluminescence (ECL) detection system (Amersham).

Flow Cytometry

After detachment with 2 mM EDTA, single-cell suspensions of 10^6 cells/ml were incubated with optimal concentrations of primary antibodies in wash buffer (2% normal goat serum in phosphate-buffered saline [PBS]) for 1 h on ice. Cells were washed three times and incubated with secondary fluorescein-labeled antibodies for 30 min on ice. After washing three times again, the cells were stained with propidium iodide (1 μg/ml) to identify nonviable cells. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson). Control samples consisted of cells with or without secondary antibody binding. Nonviable cells stained with propidium iodide were eliminated from the analysis. FACS-isolated cell lines were sorted two or three separate times.

Immunoprecipitation of Surface Biotin-labeled Cells

Cells were washed twice with PBS and then labeled with sulfosuccinimidyl-6(biotinamide)-hexanoate (NHS-LC)-biotin (Pierce, Rockford, IL), at 1 mg/ml, in PBS at 4°C for 90 min. To stop labeling, cells were washed twice with 50 mM glycine blocking buffer followed by a 10-min incubation in same buffer. Cells were lysed in PBS with 0.1 M Tris, pH 7.5, 2% Nonidet P-40, 2 mM PMSF, and 1 mM *N*-ethylmaleimide and then precleared with protein A beads. Cell lysates were mixed by rotation with primary antibody and protein A beads for ≥3 h. Beads were washed with 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40, and 0.1% bovine serum albumin (BSA) three times and heated at 100°C in SDS sample buffer for 5 min. The samples were reduced with 2-mercaptoethanol, separated by SDS-PAGE on 7.5% gels, and detected by streptavidin-HRP and ECL.

Cell Adhesion Assay

Microtiter plates (96-well Immulon plates, Dynatech, Chantilly, VA) were coated with matrix proteins or antibodies at the indicated concentrations in PBS for 1 h at 37°C in a humidified atmosphere. Plates were washed with PBS and incubated with medium containing 0.1% BSA for 60 min in a CO_2 incubator to block nonspecific adhesion. Single-cell suspensions were prepared in DMEM containing 0.1% BSA at 4×10^5 cells/ml, added in triplicate to 96-well plates, and then incubated for 30–60 min at 37°C. Nonadherent cells were removed by shaking on a titer-plate shaker (Lab-Line Instruments, Melrose Park, IL) and washed with PBS. Cells were fixed with 1% formaldehyde, stained with 1% crystal violet, and solubilized in 2% SDS; absorbance was then read at 562 nm. Cells bound to collagen (10 μg/ml) on a separate plate were used to represent 100% attachment. Background cell adhesion to 1% BSA-coated wells was subtracted from all readings. The effect of specific blocking antibodies was tested by preincubating the cells with the indicated dilutions of purified antibodies on ice for 30 min prior to the assay.

Laminin-Sepharose Affinity Chromatography

Binding of the α7 integrin on laminin E8 was performed as detailed by Kramer (1994). The E8 fragment of laminin 1 was coupled to CNBr-activated Sepharose to yield 0.5–1.0 mg/ml of packed gel. Cells were washed twice with PBS and then labeled with NHS-LC-biotin as described above. Labeled cells were then extracted with buffer containing 200 mM octyl β-D-glycopyranoside (OPG), in 50 mM Tris-HCl, pH 7.4, 1 mM $MnSO_4$, and 1 mM PMSF for 30–60 min at 4°C. The cell lysate was centrifuged first at $2000 \times g$ to remove nuclei and then at $20,000 \times g$. One milliliter of supernatant was mixed slowly by inversion with ~300 μl of packed E8-Sepharose overnight. After application of the cell extracts, the column was carefully washed with three to five column volumes of OPG buffer. Finally, the bound α7β1 complex was eluted with 10 mM EDTA followed by stabilization with excess Mg^{2+}. Fractions containing eluted integrin subunits were subjected to immunoprecipitation with the appropriate antibodies, separated in an SDS-PAGE gel under reduced and nonreduced conditions, and detected by ECL.

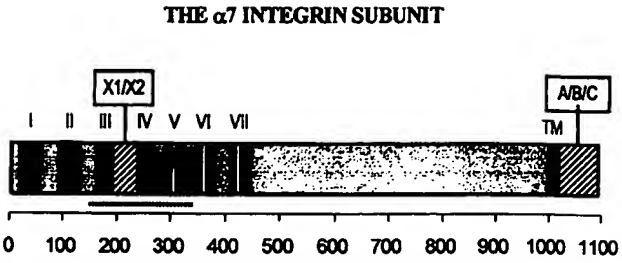

Figure 1. Structure of the α7 subunit. Diagram shows the relative positions of the identified splice sites in the extracellular domain (X1 and X2) and cytoplasmic domain (A/B/C). The seven homology repeat domains (I–VII solid boxes), the divalent cation-binding sites (open slots), the putative ligand-binding site (dotted line, residues 140–350), and the transmembrane domain (TM) are indicated.

RESULTS

Generation of α7-X1- and α7-X2-expressing Cell Lines

We previously reported that the α7 integrin subunit is a laminin-binding integrin that is alternatively spliced in the extracellular domain, producing two isoforms, designated X1 and X2 (Ziober *et al.*, 1993). This alternative splicing occurs in the variable region between the III and IV homology repeat domains near the putative ligand-binding site (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Figure 1), which could define ligand specificity, affinity, or integrin activation state. To explore for functional differences between the alternatively spliced extracellular isoforms of α7, we constructed expression vectors for α7-X1 and α7-X2. The cDNAs were then stably transfected into human MCF-7 carcinoma cells, which normally adhere poorly to laminin (Yao *et al.*, 1996b).

After selection with G418, several α7-X1- and α7-X2-expressing cell lines were isolated either by FACS sorting or by cloning rings. Two α7-X1-expressing cell lines, X1-A and X1-S3, and two α7-X2 cell lines, X2-A and X2–6, were analyzed further. FACS analysis, using the anti-α7 CY-8 mAb, verified that all cell lines expressed equivalent levels of the integrin (Table 1). In addition, Western blotting confirmed that all α7-X1 and α7-X2 MCF-7 cell lines expressed similar levels of the α7 subunit, whereas the parental MCF-7 cells were negative for the receptor (Figure 2A). Interestingly, α7-X1 displayed a slight decrease in gel mobility when compared with that of α7-X2. Molecular mass calcu-

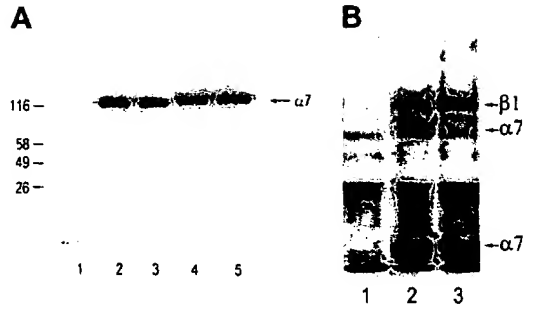

Figure 2. Expression of X1 and X2 transfectants in MCF-7 cells. MCF-7 cells were transfected with pRc/CMV-α7-X1 and pRc/CMV-α7-X2. (A) Equal quantities of cellular lysates from parental MCF-7 (lane 1), X2–6 (lane 2), X2-A (lane 3), X1-A (lane 4), and X1-S3 (lane 5) cells were processed for SDS-PAGE in a 7.5% polyacrylamide gel. After transfer, the nitrocellulose membrane was probed with anti-α7 (1211) antiserum and the position of the α7 subunit was determined by using ECL. (B) Surface-biotinylated parental MCF-7 cells (lane 1) and X1 (X1-A, lane 2) and X2 (X2-A, lane 3) transfectants were immunoprecipitated with anti-α7 polyclonal antibody 1211. Immunoprecipitates were separated by SDS-PAGE on 7.5% gels under reducing conditions to resolve the α7 subunit from comigrating β1 and transferred to nitrocellulose membranes. When reduced, the α7 subunit separates into its heavy and light chains (Yao *et al.*, 1996b). The heavy α7 chain is not strongly biotinylated (Yao *et al.*, 1996b). Proteins were visualized by incubation with streptavidin-HRP and then detected by ECL. The positions of the α7 and β1 subunits are indicated.

lations indicated that this size difference cannot be due to the four additional amino acids encoded by exon X1 as compared with exon X2 (Ziober *et al.*, 1993). This variation in electrophoretic mobility reflects an ~3300-kDa change in molecular mass between α7-X1 and α7-X2, suggesting that there is a conformational difference between the two isoforms. When samples were subjected to SDS-PAGE under reducing conditions, no differences in electrophoretic motility were detected, suggesting that the two α7-X1 and α7-X2 isoforms exist in two conformations that depend on intact disulfide bonds (our unpublished observations). Finally, immunoprecipitation of cell-surface–biotinylated cells verified that both isoforms were expressed at the cell surface and paired with the β1 integrin subunit (Figure 2B).

Table 1. Flow cytometry expression levels of α7-X1 and α7-X2 in MCF-7 cells

Cell line	Peak area[a] (arbitrary units)
X1-A	64.6 ± 7.2
X1-S3	65.2 ± 1.2
X2-A	65.8 ± 3.7
X2-6	68.9 ± 2.4

[a] Average ±SD of three experiments.

Differential Adhesion of α7-X1 and α7-X2 to Laminin

We compared the ligand binding and specificity of the α7-X1 and α7-X2 isoforms by testing the transfectants in standard adhesion assays using different concentrations of laminin 1 and other ligands. We have shown previously that parental MCF-7 cells express endogenous, potential laminin 1 receptors that are functionally inactive (Yao *et al.*, 1996b). On laminin 1, α7-X2 transfectants attached with high efficiency; adhesion was dependent on the ligand coating concentration (Figure 3A). In previous studies we have shown by using α7-blocking mAbs, that this binding to laminin 1 is specific for α7-X2-expressing MCF-7 cells (Yao *et al.*, 1996b). In contrast, the α7-X1 cell lines, like the parental MCF-7 cells, adhered poorly, even at the highest ligand concentrations. When laminin 1 was replaced with purified laminin 5, both X1 and X2 isoforms failed to bind (our unpublished observations).

The lack of adhesion of the α7-X1 cell lines to laminin 1 suggested that the transfectants might have a general defect in their ability to adhere. To evaluate this possibility, we tested the capacity of the X1 transfectants to bind collagen I. Both X1 and X2 cell lines and parental MCF-7 cells showed similar binding to collagen I, and as expected, antibodies to the α2 collagen receptor completely blocked this adhesion (Figure 3B). Binding to collagen IV showed similar results (our unpublished results).

We also evaluated whether α7-X1 or α7-X2 could bind fibronectin, because it was reported that the α7 subunit binds to this ligand (Gu *et al.*, 1994). However, both X1 and X2 transfectants bound equally to fibronectin (Figure 4). Blocking antibodies to α5 and αv dramatically decreased the ability of the X1 and X2 clones to bind fibronectin, and the combination of α5 and αv blocking antibodies totally inhibited binding to fibronectin for all three cell lines. The parental MCF-7 cells adhered to fibronectin with somewhat higher efficiency than the α7 transfectants, which presumably reflects an α7-induced decrease in the fibronectin receptor expression. We have shown a similar down-regulation of α3β1 receptor expression in MCF-7 cells transfected with α7 (Yao *et al.*, 1996b). Thus, these results show that neither the X1 nor the X2 isoform of the α7 subunit can function as a fibronectin receptor, but cells transfected with either isoform can bind collagen I and IV, indicating that the X1-transfected cells do not have a defect in adherence even though X1 does not bind laminin 1.

α7-X1 Integrin Competency for Adhesion and Spreading

The failure of the X1 isoform to bind laminin suggested that this α7 isoform may be functionally defective. To test for this possibility, we artificially cross-

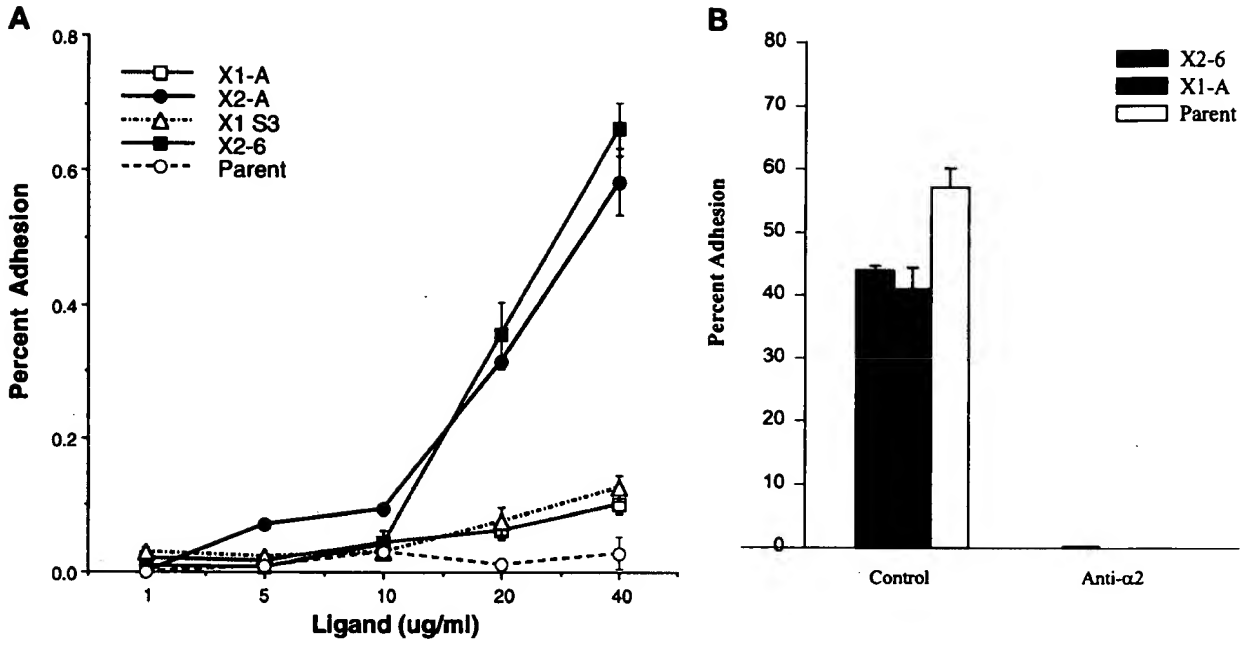

Figure 3. α7-X1 cell lines fail to bind laminin 1. (A) Dose–response curves of cell adhesion to laminin 1. Parental MCF-7, α7-X1, and α7-X2 cells (2×10^4 cells/well) were tested for adhesion to increasing concentrations of laminin 1 as described in MATERIALS AND METHODS. α7-X2 clones showed a dose-dependent adhesion to increasing concentrations of laminin 1. α7-X1 and parental cells bound poorly to laminin at all concentrations. (B) Adhesion of parental MCF-7, α7-X1, and α7-X2 cells to collagen I. Parental MCF-7, X1, and X2 cells (2×10^4 cells/well) were added to collagen-I–coated plates (5 μg/ml). Anti-α2 mAb (VM1) was preincubated with cells at 10 μg/ml. Cells bound to collagen type I (at 100 μg/ml) were used to indicate 100% adhesion. Adherence of cells in 1% BSA-coated wells was treated as background binding and subtracted. Data are presented as percentages of the total cells added to each well. (A and B) Values are the mean of triplicate wells; bars indicate the SD.

linked the α7-X1 receptor by using an anti-α7 mAb capture assay. In this assay, mAbs directed against the extracellular domain of the α7 subunit were immobilized on culture dishes (see MATERIALS AND METHODS). Transfectants expressing either α7 isoform were allowed to bind the immobilized mAbs. Both α7-X1 and α7-X2 cell lines attached with equal efficiency to the bound anti-α7 mAb (Figure 5A); when anti-β1 mAb was used, both transfectants attached somewhat more efficiently (our unpublished results). Importantly, both X1 and X2 transfectants were induced to spread within 60 min on the immobilized mAbs (Figure 5, B and C). These results show that both X2 and X1, when cross-linked by anti-α7 mAbs, induce an adhering and spreading response typical of functional integrins that requires assembly of the cytoskeleton. In addition, this suggests that although the α7-X1 integrin is unable to engage laminin, it can still function normally in postligand-binding events.

Solubilized α7-X1 Integrin Fails to Bind Ligand

To determine whether laminin binding by the two isoforms is regulated by their cellular environment, transfectants were processed for ligand affinity chromatography. Receptors were solubilized in detergent and applied to E8-laminin-Sepharose. The presence of α7 in the unbound flow-through fractions and EDTA-eluted fractions was assessed by immunoprecipitation with an α7 mAb. Nearly all of the α7-X2 bound to the E8-Sepharose column and was eluted with EDTA (Figure 6). In contrast, all of the α7-X1 was found in the flow-through and was not detected in the EDTA-eluted fractions (Figure 6). Such a result is consistent with the X1 isoform being functionally unable to engage its ligand even when freed from potential cellular regulatory elements.

The α7-X1 Isoform Can Exist in a Ligand-binding Incompetent State

The antibody-capture adhesion assay indicated that the α7-X1 isoform can function as a bridge between the cytoskeleton and the extracellular matrix. However, it fails to explain why this isoform is deficient in binding its ligand. It is well known that many integrins can alternate between activated and inactive states (reviewed in Hemler *et al.*, 1994; Humphries, 1996; Mould, 1996). This reversible transition to the

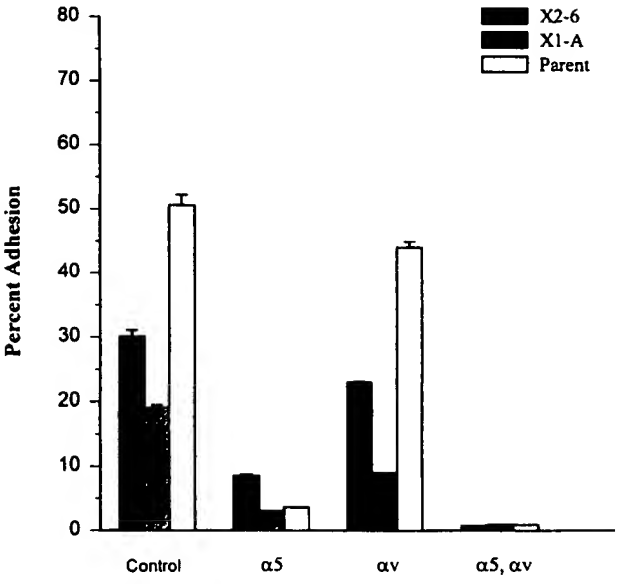

Figure 4. Adhesion of parental MCF-7, α7-X1, and α7-X2 cells to fibronectin. Parental MCF-7, X1, and X2 cells (2×10^4 cells/well) were added to fibronectin coated plates (10 μg/ml) as described under MATERIALS AND METHODS. Anti-α5 mAb (P1D6) and anti-αv mAb (LM230) were preincubated with cells at 10 μg/ml. Cell adhesion was determined as detailed in Figure 3.

active state is associated with enhanced ligand-binding affinity and is believed to depend on conformational changes that can be influenced by several factors including stimulatory or activating anti-integrin antibodies and divalent cations (Humphries, 1996). To test for the possibility that the inability of α7-X1 to bind ligand is a result of the receptor's activation state, we performed adhesion assays to laminin 1 in the presence or absence of β1 activating antibodies. Addition of the activating anti-β1 mAb TS2/16 substantially increased the adhesion to laminin 1 for α7-X1, α7-X2, and parental MCF-7 cell lines (Figure 7). The most dramatic increase occurred in the α7-X1 clone, which went from minimal adhesion in the absence of activating antibody to nearly the same level as the α7-X2 cell line in the presence of mAb TS2/16.

To identify the integrin complexes responsible for α7-X1's increased adhesion, we repeated the adhesion assays with TS2/16 and a combination of integrin-blocking antibodies. A mixture of blocking antibodies to integrin subunits α2, α3, and α6 completely inhibited TS2/16-activated MCF-7 parental cells from binding to laminin 1 (Figure 7). In contrast, these same blocking antibodies only slightly reduced the TS2/16-activated α7-X1 and α7-X2 cell adhesion to laminin, suggesting that this adhesion was mediated by the α7 subunit. When blocking antibodies to α7 were added to the anti-α2, -α3, and -α6 mAb mixture, adhesion to laminin 1 was completely inhibited for the α7-X1 and α7-X2 cell lines (Figure 7). Similar results were seen when TS2/16 was replaced with the 8A2 activating mAb but not with 9EG7 or 15/7 mAbs (our unpublished results). Results again were similar when these experiments were repeated with the laminin E8 fragment, which contains the α7 binding site (our unpublished results). Interestingly, TS2/16-activated α7-X2

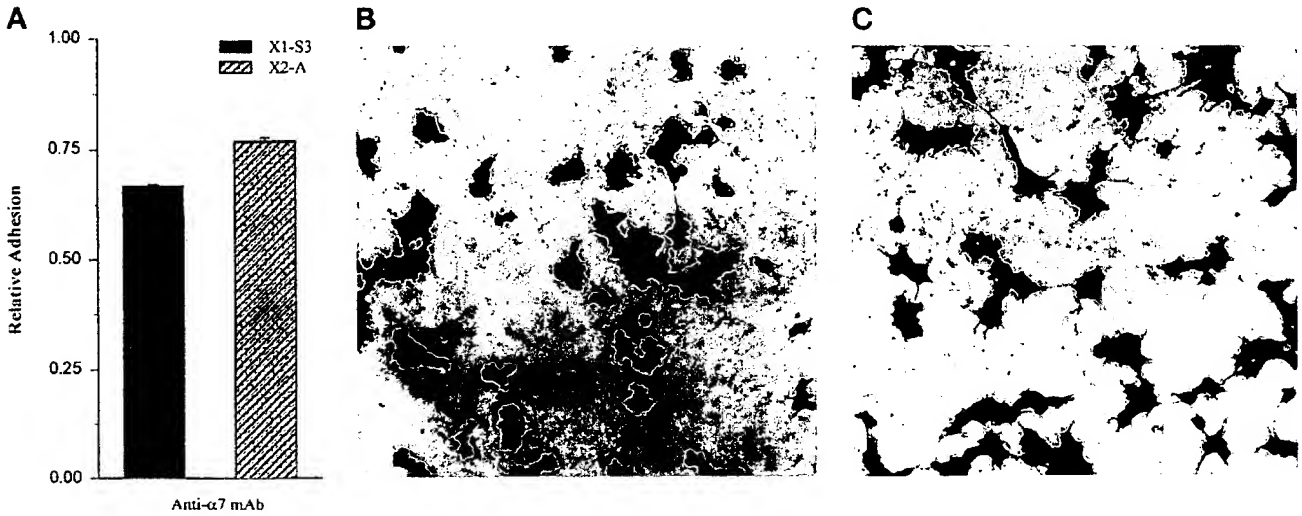

Figure 5. α7-X1 expressing cells adhere and spread on anti-α7. (A) Microtiter plates (96-well) were coated with anti-α7 mAb at 15 μg/ml in PBS. Plates were washed with PBS and incubated with DMEM containing 0.1% BSA to block nonspecific adhesion. Single cell suspensions were prepared in DMEM with 0.1% BSA; 2×10^4 cells were added to each well in triplicate in 96-well plates and incubated for 60 min at 37°C. Adherence of cells in 1% BSA-coated wells was treated as background binding and subtracted. Data are presented as relative adhesion based on optical density (OD) units. Values are the mean of triplicate wells; bars indicate the SD. Both α7-X1 (B) and α7-X2 (C) cells have attached and spread on immobilized anti-α7 mAbs. After 1 h cells were fixed with 1% formaldehyde and stained with 1% crystal violet.

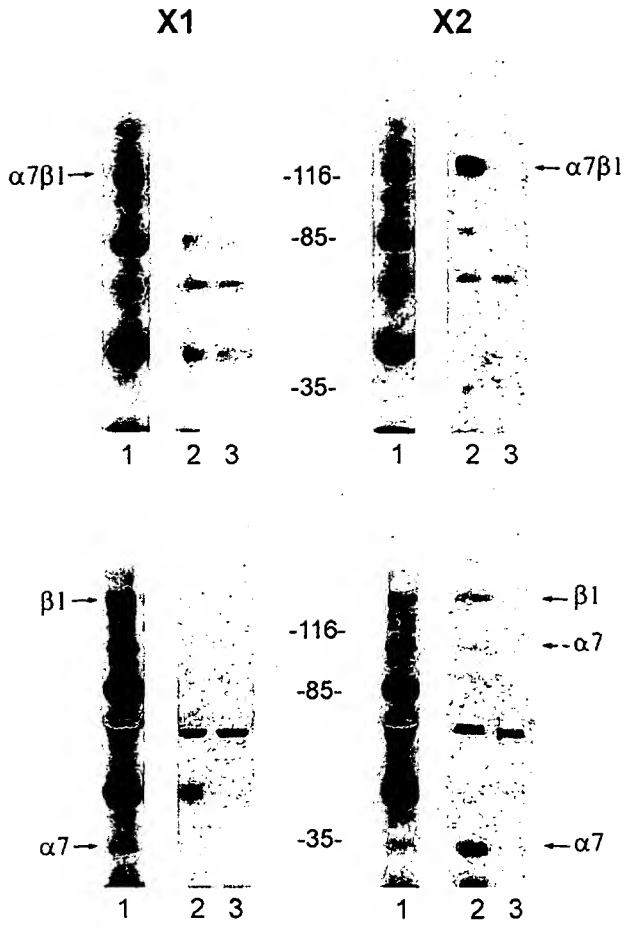

Figure 6. Detergent-solubilized α7-X2 is able to bind immobilized E8 fragment of laminin 1. Cell-surface biotin-labeled α7-X1– and α7-X2–transfected MCF-7 cells were lysed in OPG and processed for ligand affinity chromatography on laminin E8-Sepharose columns. The unbound flow-through material was collected, and after extensive washing of the column, bound integrin was eluted with EDTA (see MATERIALS AND METHODS). The material present in the crude flow-through fraction (lane 1) and the first two fractions eluted with EDTA (lanes 2 and 3) were processed for immunoprecipitation with anti-α7 antibody (1211) and analyzed by SDS-PAGE under nonreduced (top) or reduced conditions (bottom). The positions of the α7 and β1 subunits are indicated. Note that the α7 subunit separates into its heavy and light chains after reduction (Yao *et al.*, 1996b).

cells in the presence of anti-α2, -α3, and -α6 adhered at nearly the same level as the untreated α7-X2 transfectants. Thus, these results clearly demonstrate that the failure of the α7-X1 isoform to bind laminin 1 is linked to the activation state of the subunit, which in turn is regulated by the swapping of X2 and X1 inserts.

Mn^{2+} Fails to Activate α7-X1 Adhesion

Ligand binding by integrins is dependent on divalent cations (Sanchez-Mateos *et al.*, 1996). Work by a number of investigators has shown that Mn^{2+}, and to a lesser extent Mg^{2+}, can enhance ligand binding by switching the integrin to an activated state via a mechanism believed to be similar to that produced by activating β1 antibodies (Humphries, 1996; Mould, 1996). When Mn^{2+} replaced Ca^{2+}/Mg^{2+} as the divalent cation in adhesion assays, the parental, X1, and X2 cell lines all displayed an increase in adhesion to laminin (Figure 8). However, the Mn^{2+}-induced increase in adhesion seen for X1 was inhibited by a mixture of blocking antibodies to integrins α2, α3, and α6, indicating that α7-X1 is resistant to Mn^{2+}-induced activation. As with the TS2/16-treated cells, Mn^{2+} had only a slight stimulating effect on laminin 1 binding for α7-X2, suggesting that the X2 isoform is already fully activated. These results are consistent with those of the E8-Sepharose chromatography assays, which used detergent-solubilized α7 receptor in the presence of Mn^{2+}.

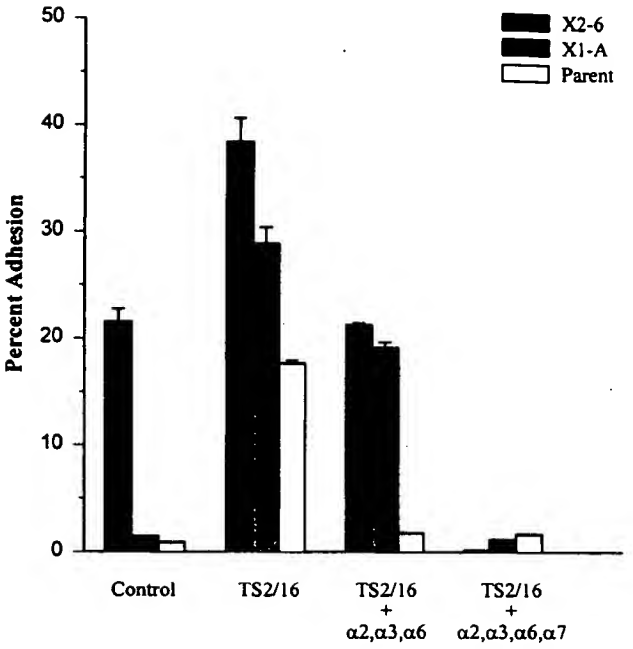

Figure 7. Stimulation of α7-X1 laminin binding activity by mAb TS2/16. (A) Parental MCF-7, X1, and X2 cells (2×10^4 cells) were added to laminin 1-coated wells (15 μg/ml) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with TS2/16 (1.5 μg/ml) and combinations of blocking antibodies α2 (VM1), α3 (P1B5), α6 (GoH3), and α7 (CY8), all at 10 μg/ml. Cell adhesion was determined as detailed in Figure 3.

Regulation of α7-X1 Ligand Competency Is Cell Type Specific

It is now appreciated that individual integrins can display altered levels of activation depending on the

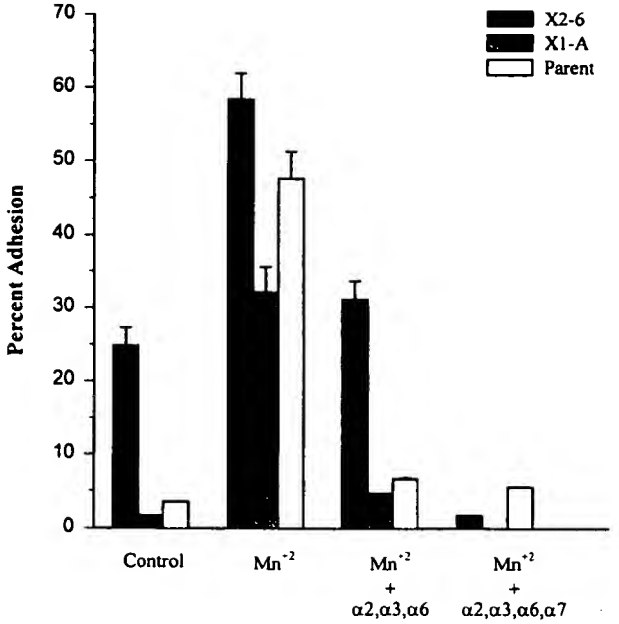

Figure 8. α7-X1 is not cation dependent for laminin 1 adhesion. Parental MCF-7, X1, and X2 cells (2×10^4 cells) were added to laminin 1-coated plates (15 μg/ml) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with Mn^{2+} (0.1 mM) and combinations of blocking antibodies α2 (VM1), α3 (P1B5), α6 (GoH3), and α7 (CY8), as in Figure 7. Cell adhesion was determined as detailed in Figure 3.

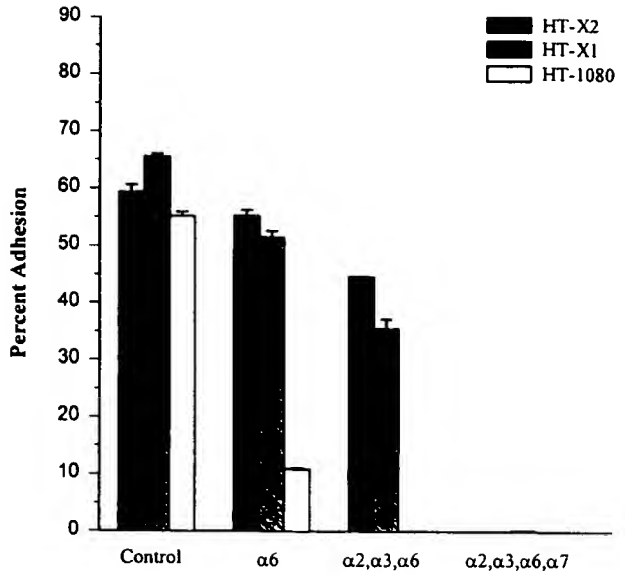

Figure 9. Adhesion of HT1080 parental and HT1080 α7-X1 and α7-X2 cell lines to laminin 1. Parental HT1080 cells were transfected with α7-X1 and α7-X2 as described in MATERIALS AND METHODS. HT1080, HT1080X1, and HT1080X2 cell lines (2×10^4 cells) were added to laminin 1-coated plates (15 μg/ml) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with combinations of blocking antibodies α2 (VM1), α3 (P1B5), α6 (GoH3), and α7 (CY8), as in Figure 7. Cell adhesion was determined as detailed in Figure 3.

cell type (Gehlsen *et al.*, 1989; Chan and Hemler, 1993). When we expressed the α7-X1 or α7-X2 variant integrins in MCF-7 cells, the two receptors showed differential binding activity for laminin 1. MCF-7 cells appear to be in the class of host cells that confer a poor level of integrin activation, as indicated by the fact that endogenously expressed α2 and α6 as well as α7-X1 integrins in MCF-7 cells are not capable of binding laminin 1, yet can be fully activated by mAb TS2/16 (Figure 7; Yao *et al.*, 1996b). For an activation-permissive cell, we chose the HT1080 human fibrosarcoma cells. This cell line expresses a fully functional α6β1 integrin, and adhesion to laminin 1 can be blocked by mAb to α6 (GoH3; Lin *et al.*, 1993; Matter and Laurie, 1994). For analysis of the cell-type-dependent activation of α7, HT1080 cells were transfected with the α7-X1 or α7-X2 isoform, and high-expressing subpopulations of each transfectant were enriched for α7 by flow cytometry after an initial selection with G418. Both the X1- and X2-transfected cell lines as well as the parent HT1080 cells showed strong adhesion to laminin (Figure 9). However, after treatment with antibodies to α6, the binding of the parental cells was greatly reduced and was completely inhibited in the presence of mAbs to α2, α3, and α6. Although the X1 and X2 transfectants also showed partial blocking of adhesion to laminin in the presence of this mixture of mAbs, substantial binding was still evident, but this binding was abolished when α7 mAb was included. This indicates that in contrast to α7-X1 expression in MCF-7 cells, the integrin's expression in HT1080 cells leads to activation levels that are nearly equivalent to those of the α7-X2 variant.

DISCUSSION

In this study we investigated possible functional differences between X1 and X2, two variants of the α7 integrin subunit that are generated by alternative mRNA splicing in the extracellular domain of the receptor. Our findings show that the alternatively spliced X1 sequence modifies the activation state of the integrin. Whereas the α7-X2 isoform is constitutively active in both cell types, the X1 variant is inactive in MCF-7 cells and fully active in HT1080 cells. This is the first example of cell-type regulation of integrin function that is controlled by alternative splicing in the extracellular subunit domain.

Several α integrin subunits can be alternatively spliced in the extracellular domain between the III and IV N-terminal repeat domains, a region critically important for ligand binding (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Mould, 1996). Alternative

splicing in *Drosophila* integrin αPS2 has been shown to alter the binding efficiency of the receptor to the RGD-dependent ligands fibronectin and vitronectin (Brown *et al.*, 1989; Zavortink *et al.*, 1993; Roote and Zusman, 1996). The α6 subunit is also alternatively spliced; however, unlike α7, alternative splicing in α6 results in either exon X1 or a combination of both exons X1 and X2 being spliced in. In contrast to α7, the X1 and X1X2 isoforms of α6 showed no difference in ligand affinity, specificity, or integrin activation. Furthermore, α6-X1X2 is found as a rare mRNA and is probably not physiologically relevant, whereas expression of the α6-X2 isoform has not been detected (Delwel *et al.*, 1995). Loss of α6-X2 expression probably reflects an evolutionary change in the primordial α7-like gene that is believed to have given rise to α6. It has been suggested that the α3 subunit is also alternatively spliced extracellularly, but the X1 isoform for this subunit has yet to be described (Ziober *et al.*, 1993). In contrast to α6, both α7 alternatively spliced isoforms X1 and X2 are expressed (Ziober *et al.*, 1993; Wang *et al.*, 1995). More importantly, this alternative splicing, as shown herein, regulates the α7 receptor's ability to bind ligand.

Since α7-X1 lacks detectable laminin 1 binding activity, we postulated that the α7-X1 variant is expressed in an inactive but presumably reversible conformation, an event that can occur in other integrins. Furthermore, it is now appreciated that individual integrins can display altered levels of activation that are dependent on cell-type specificity (Hemler *et al.*, 1994). When we expressed the α7-X1 or α7-X2 variant integrins in MCF-7 cells, they showed differential binding activity for laminin 1 (Figure 3A). Endogenously expressed α2 and α6 integrins are not capable of binding laminin 1, indicating that MCF-7 cells appear to be a class of host cells that confers a poor level of integrin activation (Yao *et al.*, 1996b). These two integrins also behave similarly when transfected into the nonpermissive K562 cells (Chan and Hemler, 1993; Delwel *et al.*, 1993, 1995; Kawaguchi and Hemler, 1993). In contrast, the α3 subunit (the X2 form), when transfected into K562 cells, is constitutively active for binding laminin 5, as is endogenous α3 in MCF-7 cells (Weitzman *et al.*, 1993; Delwel *et al.*, 1994). This indicates that the α3 integrin is constitutively active even in cells such as K562 and MCF-7. Thus we consider the α3 integrin to be similar to α7-X2 with regard to its activation state and its sequence homology at the III/IV variable region. In contrast, we suggest that the normally expressed form of α6, the α6-X1 homologue, is cell type specific for activation because of its high homology to α7-X1. HT1080 human fibrosarcoma cells appear to be an X1-activation–permissive cell line because they express constitutively activated α6β1. Thus, α7-X1 and α7-X2, when transfected into HT1080 cells, were both active, indicating that activation of the X1 isoform, either for α6 or α7, depends on cell-type specificity (Figure 9).

The functional state of an integrin complex is dependent on the conformation of its extracellular domain. A number of factors have been shown to modulate the activity of this extracellular conformation (Humphries, 1996; Mould, 1996). For example, integrins are sensitive to divalent cation occupancy, and it is well established that cations can regulate integrin activity (Humphries, 1996). In particular, low concentrations of Mn^{2+} have been shown to induce a high ligand affinity state in integrins (Schwartz *et al.*, 1995). In our studies with the MCF-7 transfectants, Mn^{2+} was able to activate only the α2 and α3 integrins and not the α7-X1 isoform. However, Mn^{2+} did not appear to elevate X2 activity, suggesting that Ca^{2+}/Mg^{2+} are sufficient for a fully functional α7. This suggests that insertion of the X1 exon appears to alter the conformation of the subunit (which probably accounts for the ~3300-kDa molecular mass change in X1 as compared with X2, Figure 2). Such a conformational change may possibly affect cation binding. Similar studies with α2 showed that cations could not convert the VLA-2 form-O or form-C into a more active form but activating antibodies could (Chan and Hemler, 1993).

In general, activating antibodies appear to increase the ligand binding competency of integrin molecules. We have shown in this study that the X1 isoform is competent to bind laminin 1 only when activated by mAbs TS2/16 or 8A2 (Figure 7; our unpublished results). TS2/16 and 8A2 bind to the same epitope and activate the β1 integrin subunit by altering its conformation (Faull *et al.*, 1993; Humphries, 1996). In contrast, 9EG7 was not able to induce an active conformation in the X1 isoform (our unpublished results). This antibody recognizes an epitope, distinct from that of TS2/16 and 8A2, that is present when β1 is activated by Mn^{2+} (Bazzoni *et al.*, 1995; Humphries, 1996). 9EG7 is believed not to define an activation epitope but a ligand-induced binding site that can be induced by cations like Mn^{2+} (Bazzoni *et al.*, 1995). Perhaps the failure of 9EG7 to activate the α7-X1 isoform is also due to this isoform's inability to be activated by Mn^{2+}. Antibody 15/7 also failed to activate α7-X1; it too is considered a Mn^{2+}-inducible anti-β1 antibody (Picker *et al.*, 1993; Puzon-McLaughlin *et al.*, 1996). These results and the failure of X1 to bind laminin 1 in the presence of Mn^{2+} support the notion that the X1 segment is able to alter the conformation of the subunit and inhibit cation binding and ligand adherence.

Comparison of the X1 and X2 alternative splice domains indicates a completely divergent amino acid sequence (Ziober *et al.*, 1993). Recent observations have indicated that critical residues for ligand-binding activity are clustered in a predicted β-turn of the C-terminal portion of the third repeat domain of inte-

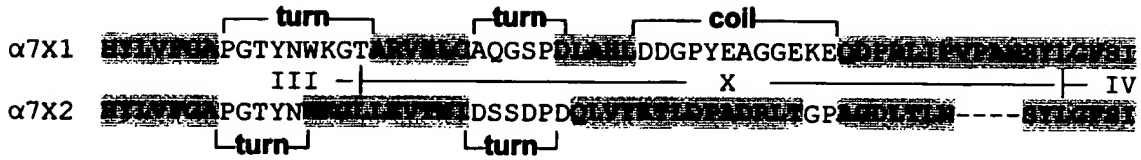

Figure 10. Amino acid sequence and secondary structure of the α7-X1 and X2 alternatively spliced domains. The amino acid sequences for X1 and X2 domains were analyzed by using the MacVector program. β-sheet conformations are boxed and β-turn or random coil regions are indicated. Both X1 and X2 have extensive β-sheet structures; however, X1 has a long random coil motif absent in the X2 that could act as a hinge.

grins α4, α5, and αIIb (Irie *et al.*, 1995; Kamata *et al.*, 1995). This site (GAPGTYNWKG) is conserved in all α-chains, including α7, and is immediately proximal to the α7 X1/X2 domains. This points to the possibility that the region defined by X1/X2, which directly borders the third repeat domain, may be important in regulating integrin activation. The β-turn at this site has been predicted by using several secondary structure prediction methods and from the alignment of the seven repeat domains from 16 different integrin sequences (Tuckwell *et al.*, 1994). Comparison of the α7-X1 and α7-X2 sequences shows a significant structural difference in the C-terminal half of the variable region (Figure 10). Although the X2 region seems to consist of multiple β-sheet structures that apparently align with β-sheet segments in the adjacent IV repeat domain, the X1 region contains a random coil structure of at least 10 residues that begin in the C-terminal half of this variable region. This coil motif in X1 may provide a "hinge-like" structure that may control conformational changes in this putative ligand-binding region of the α7 subunit (Figure 10). Such regulation may ultimately affect ligand-binding competency by turning on or off the subunit's ability to bind to divalent cations. Finally, as shown in this report, this apparent hinge-like structure can be modulated by β1-activating antibodies such as TS2/16.

How is the activation level of the α7 variants regulated? The observation that β1 activation mAbs can convert the α7-X1 isoform to the "on" state suggests that ligand-binding activity can be controlled by alterations in the partner subunit conformation. Affinity modulation by inside-out signaling via transmembrane conformational transitions has been observed previously (reviewed in Schwartz *et al.*, 1995). Since the α7 subunit expression is limited to a few highly differentiated tissues and the expression of the X1/X2 isoforms is developmentally regulated, it is possible that the functionality of this integrin needs to be controlled in a tissue-specific manner. Previous work has shown that α7β1 is expressed during myoblast differentiation and at the myotendinous and neuromuscular junctions in adult skeletal muscle (Song *et al.*, 1992; Ziober *et al.*, 1993; Martin *et al.*, 1996). Both isoforms are expressed in myoblasts, but only X2 is detectable in mature muscle, implying that the constitutively active form of the receptor is present at permanent myotendinous junctions and neuromuscular sites (Song *et al.*, 1992; Ziober *et al.*, 1993; Crawley *et al.*, 1996; Martin *et al.*, 1996). We propose that the regulatable X1 isoform is important during dynamic adhesion situations related to muscle development (motility, fusion, remodeling, repair, and matrix assembly) and that the X2 variant performs more stable adhesion functions (costamers and myotendinous junctions). The function of the two isoforms in skeletal myoblast/myotube adhesion is currently under investigation. Finally, the ras/raf/MAPK signaling pathway has lately been implicated in regulating the integrin affinity state (Hughes *et al.*, 1997). Whether this pathway plays an "inside-out" role in regulating the X1 isoform or even in cell-specific activation of this isoform remains to be determined.

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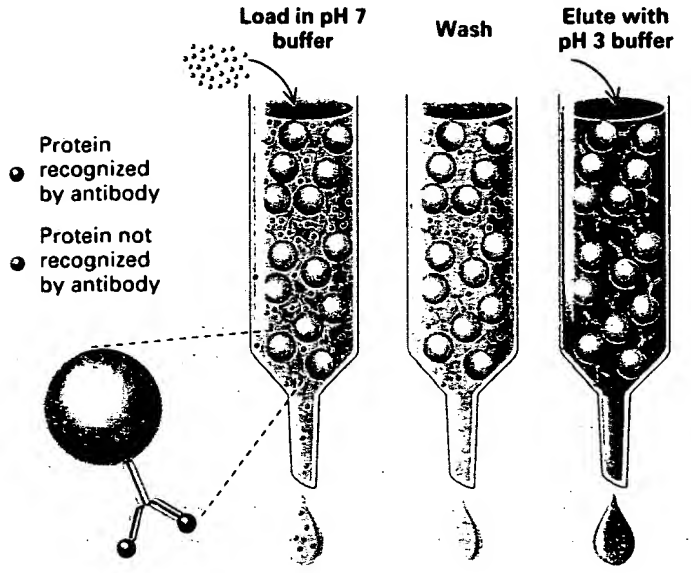


▲ FIGURE 3-34 Purification of a protein from a mixture by antibody-affinity chromatography. The mixture first is filtered through a column consisting of small beads coated with antibody molecules that are specific for the desired protein. Only that protein binds to the antibody matrix; any other proteins in the mixture pass through the column. To elute the bound protein, an acidic solution is added to disrupt the antigen-antibody complexes; the pure protein then is collected from the bottom of the column.

catalytic activity. A key difference between enzymes and antibodies, as pointed out by Linus Pauling, is that enzymes bind the strained transition-state conformation of molecules, whereas antibodies bind the ground state, or normal conformation, of molecules. What happens if an antibody is tricked into binding the transition-state conformation of a molecule? As anticipated, the antibody now can act like an enzyme.

Such enzymes, called *catalytic antibodies*, have been produced by protein-engineering techniques (Chapter 7) and selection of antibodies that bind stable analogs of transition-state intermediates; such an analog resembles the normal transition-state intermediate of a reaction. By binding selectively to the transition state, catalytic antibodies, like enzymes, lower the free energy of the transition state, thereby increasing the rate of the reaction. In the earliest experiments, antibodies were generated against a phosphonate analog (Figure 3-35). This analog is a stable phosphorous-containing compound that resembles the tetrahedryl transition state of esters as they are hydrolyzed. Although made against phosphonates, these catalytic antibodies selectively hydrolysed esters at a rate several thousand-fold faster than control nonester molecules. Because antibodies against nearly any molecule can be generated, this special use of antibodies opens new opportunities in medicine and industry.

➤ *Techniques for Purifying and Characterizing Proteins*

A protein must be purified before its structure and the mechanism of its action can be studied. However, because proteins vary in size, charge, and water solubility, no single method can be used to isolate all proteins. To isolate one particular protein from the estimated 10,000 different proteins in a cell is a daunting task that requires methods both for *separating* proteins and for *detecting* the presence of specific proteins.

Any molecule, whether protein, carbohydrate, or nucleic acid, can be separated from other molecules based on large differences in some physical characteristic. Although the sequence of amino acids in a protein uniquely determines its function, the most useful physical characteristic for separation of proteins is *size*, defined as either length or mass. Therefore, the size of protein molecules, and other polymers such as RNA or DNA, is one of the most frequent measurements in molecular cell biology. In this section, we briefly outline different techniques for separating proteins based on their size and/or other properties. The newer techniques are so simple and effective that they may not be appreciated as "physics in action." These techniques also apply to the separation of nucleic acids and other biomolecules. Finally, we consider general methods for detecting, or *assaying*, specific proteins.

Centrifugation Can Separate Particles and Molecules That Differ in Mass or Density

The first step in a typical protein-purification scheme is centrifugation. The principle behind centrifugation is that two particles in suspension (cells, organelles, or molecules) having different masses or densities will settle to the bottom of a tube at different rates. Remember, mass is the weight of a sample (measured in *grams*), whereas density is the ratio of its weight to volume (*grams/liter*). Proteins vary greatly in mass but not in density. The average density of a protein is 1.37 g/l. Unless a protein has an attached lipid or carbohydrate, its density will not vary by more than 15 percent from this value. Table 3-1 lists the density and other physical characteristics of blood proteins. You can quickly sense the differences among proteins from this table. Heavier or more dense molecules sediment more quickly than lighter or less dense molecules; with time, a pellet of molecules forms at the bottom of the tube. The remaining liquid, the supernatant, contains the nonpelleted material.

A centrifuge is an instrument that speeds this process by subjecting the particles to centrifugal forces as great as 600,000 times the force of gravity *g*. The centrifugal force is proportional to the rotation rate of the rotor (measured in revolutions per minute, or rpm) and the distance of the tube from the center of the rotor. Modern ultracentrifuges

teins with a net negative charge (acidic proteins) adhere to the beads; neutral and basic proteins flow unimpeded through the column (Figure 3-39b). The acidic proteins are then eluted selectively by passing a gradient of increasing concentrations of salt through the column. At low salt concentrations, protein molecules and beads are attracted by their opposite charges. At higher salt concentrations, negative salt ions bind to the positively charged beads, displacing the negatively charged proteins. In a gradient of increasing salt concentration, weakly charged proteins are eluted first and highly charged proteins are eluted last. Similarly, a negatively charged column can be used to retain and fractionate positively charged (basic) proteins.

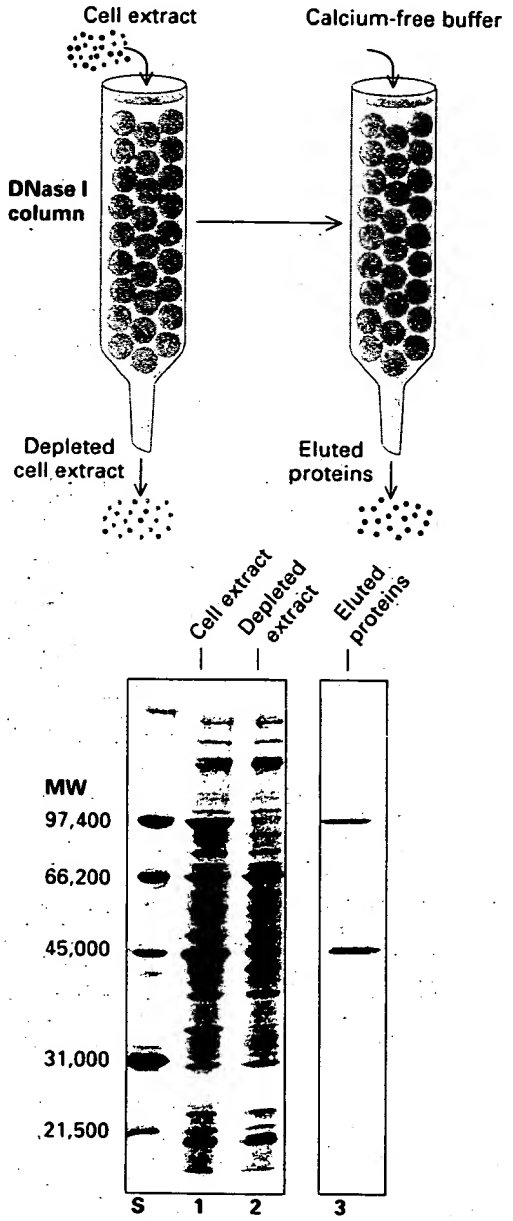
Affinity Chromatography A third form of chromatography, called affinity chromatography, relies on the ability of a protein to bind specifically to another molecule. As illustrated in Figure 3-34, an antibody can be used as an affinity reagent to isolate its corresponding antigenic protein from a mixture of proteins. Similarly, various small molecules, such as substrates for enzymes and ligands for other proteins, can be covalently attached to beads. Columns packed with such beads will retain only the proteins that bind the small molecule; the remaining proteins, regardless of their charge or mass, will pass through the column without binding to it (Figure 3-39c). The proteins bound to the column then are eluted by adding an excess of ligand, or by changing the salt concentration or pH.

By ingenious application of affinity chromatography, a wide variety of proteins can be purified in just a few steps from complex protein mixtures. In the example shown in Figure 3-40, the cytoskeletal proteins, actin and villin, form a complex which is specifically separated from an extract of intestinal cells containing hundreds of proteins by use of a DNase I affinity column. This affinity separation purifies two proteins from a cell extract in one step.

Highly Specific Assays Can Detect Individual Proteins

Purification of a protein, or any other molecule, requires a specific assay that can detect the molecule of interest in column fractions or gel bands. An assay capitalizes on some highly distinctive characteristic of a protein: the ability to bind a particular ligand, to catalyze a particular reaction, or to be recognized by a specific antibody. An assay must also be simple and fast in order to minimize errors and the possibility that the protein of interest is denatured or degraded while the assay is performed. The goal of any purification scheme is to isolate sufficient amounts of a given protein for study; thus a useful assay must also be sensitive enough that only a small proportion of the available material is consumed. Many common protein assays require just 10^{-9} to 10^{-12} g of material.

Chromogenic Enzyme Reactions Many assays are tailored to detect some functional aspect of a protein. For



▲ FIGURE 3-40 Purification of actin and villin from an extract of intestinal cells by affinity chromatography. *(Top)* In the presence of calcium, proteins actin and villin form a complex that binds to an affinity column made from beads coated with DNase I. The bound proteins can be eluted with a calcium-free buffer. *(Bottom)* SDS gel electrophoresis demonstrates separation achieved with the DNase I column. The original cell extract (lane 1) is depleted of two proteins (with molecular weights of about 95,000 and 42,000) after passage through the column (lane 2). The fraction eluted from the column by calcium-free buffer contains two proteins, actin and villin (lane 3). Lane S is of protein standards with known molecular weights. [Photograph courtesy of Mark Chafel.]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	Examiner: Maher Haddad
Donald Gullberg	Art Unit: 1644
Serial No. 09/980,403	
Filed: April 15, 2002	
For: "Integrin Heterodimer And An Alpha Subunit Thereof"	

DECLARATION OF PROFESSOR DONALD GULLBERG

I, Professor Donald Gullberg, hereby declare that:

1. I am a Professor of Biomedicine at the University of Bergen, Norway.

2. My *curriculum vitae* is provided as Appendix 1.

3. I am the sole inventor in respect of US Patent Application No. 09/980,403, which relates to the human integrin alpha-11 subunit and its use.

4. I have reviewed the examination report dated 19 May 2005 issued in connection with US 09/980,403, including the references cited therein.

5. I have been asked to comment on the following scientific paper, on which I am named as the first author:

   Gullberg et al. 1995, *Dev. Dynamics* 204, 57-65

6. My paper describes the purification of a novel integrin alpha chain, which we designated alpha-mt, from human fetal myotubes.

7. The examiner asserts that the integrin alpha-mt subunit is identical to the alpha-11 subunit described in US 09/980,403. In support of this assertion, the examiner has cited the passage at page 25, lines 32 to 37 of US 09/980,403, wherein it

is stated that '*the present data show that alpha-11 integrin is identical with alpha-mt*'.

8. However, this conclusion is based on the physical properties of the alpha subunits and their *in vitro* expression, not on their amino acid sequence. This is important because the polypeptides claimed in US 09/980,403 are limited by reference to their amino acid sequence, namely SEQ ID NO: 2.

9. We have never been able to sequence the alpha-mt gene and determine the amino acid sequence of the encoded protein. In fact, to the best of my knowledge, the amino acid sequence of the alpha-mt subunit described in the Gullberg *et al.* paper has never been determined.

10. Hence, it is not possible to conclude that the alpha-11 subunit and the alpha-mt subunit are identical at the level of amino acid sequence.

11. Consequently, the examiner's assertion that the amino acid sequence of SEQ ID NO: 2 is an inherent feature of the alpha-mt subunit is lacking scientific credibility.

12. In fact, we attempted to clone the alpha-mt gene, as described in the Gullberg *et al.* paper, from human fetal G6 myotubes but did not succeed. It was only after we decided to use a different cDNA library to that used in the Gullberg *et al.* paper, namely a human *uterus* cDNA library, that we succeeded in cloning the alpha-11 gene.

13. Given that we used a different source material for cloning the alpha-11 gene, it is impossible to conclude that the alpha-mt subunit disclosed in the Gullberg *et al.* paper comprises an identical sequence to that of the alpha-11 subunit the present application. For example, one of several different possibilities is that the alpha-11 and alpha-mt subunits are homologues or allelic variants with one or more amino acid differences. However, such homologues or allelic variants would not comprise an amino acid sequence identical to that shown in SEQ ID NO: 2.

14. The Examiner supports his novelty objection with reference to a paper by Velling *et al.*, 1999, *J. Biol. Chem.* 274:25735-25742. This paper, on which I am also named as an author, corresponds to the

disclosure in US 09/980,403 but was published after its filing date. Again, comparisons in this paper between the integrin alpha-mt subunit and the alpha-11 subunit described in US 09/980,403 are based on the physical properties of the alpha subunits and their *in vitro* expression, not on their amino acid sequence. Hence, it cannot be concluded that the alpha-mt subunit comprises the amino acid sequence of SEQ ID NO: 2.

15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the above-referenced application or any patent issued thereon.

Professor Donald Gullberg

Nov. 10th, 2005
Date
Bergen, Norway

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	)	Examiner: Maher Haddad
	)	
Donald Gullberg	)	Art Unit: 1644
	)	
Serial No. 09/980,403	)	
	)	
Filed: April 15, 2002	)	
	)	
For: "Integrin Heterodimer And An Alpha Subunit Thereof"	)	

DECLARATION OF DR TEET VELLING

I, Dr Teet Velling, hereby make the following declaration:

1. I am a research scientist at the Department of Medical Sciences, Uppsala University, Uppsala, Sweden

2. My *curriculum vitae* is provided as Enclosure A.

3. I have read and am familiar with US Patent Application No. 09/980,403, which concerns the human integrin alpha11 subunit and its use. I have also reviewed the examination report dated 19 May 2005 issued in connection with US 09/980,403, including the references cited by the examiner.

4. My comments on the merits of the objections raised by the examiner are as follows.

5. The examiner has raised an objection based on the paper by Gullberg et al. 1995, Dev. Dynamics 204, 57-65. I am familiar with this paper as I was involved in the work described in it (and I am named as an author). The paper describes the upregulation of the integrin alphamt subunit on human fetal myotubes.

6. The examiner alleges that the alphamt subunit is identical to the alpha11 subunit described in US 09/980,403. In support of this allegation, the examiner also cites a later paper which I authored, Velling et al., 1999, J. Biol. Chem. 274, 25735-25742.

7. The experiments in the Velling et al paper correspond to the experiments described in the Examples section of US 09/980,403. The inventor, Donald Gullberg, is also an author on this paper.

8. In alleging that the alphamt subunit is "identical" to the alpha11 subunit, the examiner has failed to define what he means by the term "identical". It is my understanding that under patent law, the issue of novelty requires 100% *sequence* identity between the two alpha subunits.

9. The basis of this comparison is highly significant because the alphamt subunit has never been sequenced. It is therefore impossible to conclude that the alphamt subunit shares 100% sequence identity with the alpha11 subunit; this comparison has never been done.

10. One could speculate that the amino acid sequences of the alphamt subunit will be similar to the alpha11 subunit, based on the cloning strategy employed. However, it is simply not possible to say that the amino acid sequence are 100% identical.

11. In my opinion, it is not even possible to say that 100% sequence identity between the alphamt subunit and the alpha11 subunit is *probable* since the two subunits were derived from different tissues. The alphamt subunit is derived from muscle cell precursors (myotubes) while the alpha11 subunit is cloned from a uterus cDNA library. It is quite possible that there will be a degree of sequence variation in such proteins expressed by different tissues.

12. As a consequence, I believe that the examiner's objection based on Gullberg et al. is lacking in scientific basis.

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the above-referenced application or any patent issued thereon.

T Velling

Dr Teet Velling

15/11-05

Date